STUDIES IN VIRUS DISEASES.

T H E S I S

submitted for the degree of

Doctor of Science

University of Edinburgh

by

C. E. van ROOYEN, M.D.
Major R.A.M.C.

1941.

PART I.

TECHNICAL METHODS EMPLOYED IN THE STUDY OF VIRUS ELEMENTARY BODIES, WITH SPECIAL REFERENCE TO THE AGENTS OF INFECTIOUS MYXOMATOSIS OF THE RABBIT AND TO RIFT VALLEY FEVER.

As long ago as 1887 John Brown Buist, a graduate of Edinburgh University, published a monograph in which he stated that the infective agents of variola and vaccinia were minute bodies, much smaller than the pyogenic cocci found in vaccinial pustules. Buist's historical studies were performed in a small bacteriological laboratory at the University attached to the Department of Surgery under the direction of Professor Cheyne, and it is of particular interest to mention that this laboratory, which was instituted in 1882, was probably the first laboratory of its kind in Great Britain.

Unhappily Buist's work failed to receive the credit it deserved, and it was not until 1937 that Mervyn Gordon drew attention to the priority of Buist's claims in the discovery of the virus elementary body. In 1906, Paschen of Hamburg working along similar lines to those of Buist also observed that the virus of variola-vaccinia was a minute structure which he named the elementary body, and which term has subsequently been retained in the literature. During the last fifteen years the aetiology of virus diseases has been intensively investigated and the efforts of physicists/

physicists, chemists, crystalographers and others have been united together in an attempt to solve some of the more difficult problems which have arisen. It would serve no useful purpose to reiterate the sequence of scientific events which have culminated in the present state of our knowledge regarding virus bodies, but in general terms it can be said that progress has occurred along two lines of investigation, namely, by the direct study of virus morphology by microscopy, and from indirect evidence of size and other properties by means of filtration and centrifugation experiments.

Many different types of elaborate and expensive equipment have been devised for this class of research and apparatus such as the U-V light, electron, and fluorescence microscopes have been invented. Much, nevertheless, can be accomplished with every day optical equipment and in my work I have tried to show how many of the so-called ultra-microscopic and invisible viruses can be studied with advantage by the ordinary microscope, and I have described certain technical features which should prove of assistance to others engaged in the examination of pathological tissues for elementary bodies.

In any approach to this subject it is essential first to review the relevant literature, and secondly to examine and discuss the physical, chemical and physico-chemical principles upon which instruments such/

such as the ultra violet light, electron, and fluorescence microscopes are constructed. Only in this way can one interpret the significance of published research and correlate them with one's own deductions.

The following pages contain a comprehensive description of apparatus used, their mode of operation, scope of usefulness and methods of interpreting data. The text is subdivided into sections dealing with microscopy, staining, filtration and centrifugation, in which are contained personal observations on the morphology of the elementary bodies of myxoma and their centrifugalisation, also an account of the existence of elementary bodies in Rift Valley fever.

CHAPTER I

THE MICROSCOPICAL EXAMINATION OF ELEMENTARY BODIES

THE USE OF THE ORDINARY MICROSCOPE FOR THE DEMONSTRATION OF ELEMENTARY BODIES

THE detection of elementary bodies has become a matter of considerable importance in the recognition of certain virus diseases, and in recent years these bodies have been demonstrated in, for example, vaccinia, variola, ectromelia, herpes, varicella, psittacosis, fowl-pox, canary-pox, infectious myxomatosis, and fibromatosis of rabbits. The value of the ordinary microscope for this purpose has been somewhat underrated.

The limit of visibility is stated by Coles (1929) to be 0·074 μ with ordinary white light and 0·0673 μ with green light. The macula of the retina is most sensitive to green light and, owing to the additional contrast which is imparted, a smaller object becomes more easily visible with green than with white light. It must be borne in mind that the employment of mordants for staining elementary bodies increases their size, so that although vaccinia virus may actually be 0·15 μ in diameter by ultra-filtration measurements, after staining it may appear to be 0·3 μ. With improvements in technique it is possible that viruses even smaller than 0·067 μ may be seen as stained particles. There are indications that further developments are likely to occur along these lines, and recently Merling-Eisenberg (1938), by taking advantage of the fact that the limit of visibility is greater in the dark field, has succeeded in photographing the particles of a *B. coli* bacteriophage which only measured 0·025 μ in diameter. A particle of such size lies on the border-line of visibility, and great skill and care are required to differentiate between virus particles and artefacts. The limit of visibility mentioned above must not be confused with the limit of resolution. The latter is generally stated to be 0·25 μ with direct light: this figure represents the smallest space that could be appreciated by the eye if two objects were placed near to each other. For example, if two parallel lines were gradually brought closer together, as soon as they were less than 0·25 μ apart it would be impossible to discern the gap existing between them and this distance would represent the limit of resolution. Thus, the two images would seem to have fused together and appear as a single straight line; according to Johnson (1928), an objective possessing a numerical aperture of 1·4 is able to resolve a space of 0·2 μ or separate 140,000 lines per inch.

When employing oblique illumination, the limit of resolution or separation is smaller still, and two refractile dots, viewed in a dark field, can be separated if they are situated less than 0·2 μ apart, and by means of dark-field illumination phage particles of the order of 25 mμ in size have been photographed by Merling-Eisenberg (1938).

Whilst the limit of resolution with a lens of 1·4 N.A. is about 0·25 μ, the limit of visibility of this glass when used at full aperture is 0·074 μ for a deeply stained particle seen with ordinary white light, and 0·0673 μ for one viewed in green light (Coles, 1929).

The resolving power of a lens has been theoretically explained by Martin and Johnson (1931). Owing to the wave nature of light, an objective is unable to reproduce a sharply defined outline of a minute brightly

illuminated object; instead of which it produces an image that consists of a central bright spot surrounded by a series of concentric diffraction rings. This phenomenon has been called the Airy (1834) disk, the radius of which (h') is deduced according to the formula $h' = \frac{0{\cdot}61\lambda}{\sin U'}$, when $\lambda =$ the wave-length of light employed and $U' =$ the angle between the marginal rays of the lens and its optical axis. Hence, if two point objects be brought closer towards each other, it can be proved by means of an intensity curve drawing that the two images will be separated by a dark zone only at such a point when their centres are situated at a distance which

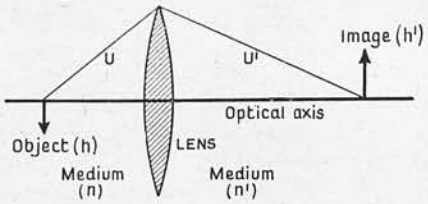


FIG. 1. Diagram to explain formation of Airy disk.

is at least approximately equal to the radius of the Airy disk (see Martin, 1925–6).

By application of the sine relation to an optical system it can be shown from Fig. 1 that $nh \sin U = n'h' \sin U'$, where n and n' represent the refractive indices of the media lying on the object and image sides of the lens and U and U' the angles subtended by the marginal ray and the optical axis. In order to find the smallest distance between two objects conditional with resolution, h' must be substituted by the value of the radius of the Airy disk which is $\frac{0{\cdot}61\lambda}{\sin U'}$, and accordingly

$$nh \sin U = n' \times \frac{0{\cdot}61\lambda}{\sin U'} \times \sin U'.$$

But since the refractive index of the medium in which the image is formed is air and this is equal to unity, $h = \frac{0{\cdot}61\lambda}{n \sin U'}$. But $n \sin U$ is equal to the numerical aperture (N.A.) of the lens (see Mackie and McCartney, 1938, for explanation), therefore $h = \frac{0{\cdot}61\lambda}{\text{N.A.}}$. It can accordingly be stated that the resolving power of an objective is directly proportional to the wave-length of light employed and varies inversely with the numerical aperture of the lens. In the following pages, with the help of a diagram, we shall describe a simple method by which the limit of visibility can be proved to be smaller than the limit of resolution.

The Limits of Visibility and Resolution Diagrammatically Explained

We have just mentioned that when using direct light the limit of visibility is smaller than the limit of resolution. In other words, it is easier for the human eye to perceive a deeply stained particle than to discern a space of the same size lying between two parallel lines. This fact can be demonstrated graphically by means of a scale drawing. Let us presume that Fig. 2 represents a microscopic field viewed under $\times 1{,}000$ magnification, showing a series of circular black dots, the largest of which

measures 1 μ and the smallest 0·1 μ in diameter. Adjacent to each of these is a pair of parallel lines separated by a distance equal to the diameter of the circle opposite to it. Thus, the gap between the first and widest pair of lines measures 1 μ and the last and narrowest is 0·1 μ.

A glance at the range of black spots, which may be assumed to represent deeply stained particles in a microscopic field, will immediately show that, whereas a minute black circle of 0·4 μ is readily visible, it is perhaps not so easy to see a gap of 0·4 μ between the corresponding parallel lines. The next pair of objects makes this point more clear, for it will be observed that, although a particle of 0·2 μ in diameter can still be distinctly

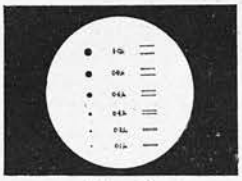


FIG. 2.

FIG. 3.

FIG. 2. For the interest of the reader we should like to state that the above minified illustration was prepared from an accurate scale drawing made on graph paper in which the largest circle measured 1 centimetre and the smallest 1 millimetre. The diagram was then reduced ten times so that the largest circle became 1 mm. and the smallest 0·1 mm. The drawing has been placed in a circular mask in order to convey the impression seen in a $\times$1,000 microscopical field in which the largest circle was 1 μ in diameter. The distance between each set of parallel lines corresponds to the size of the adjacent circles.

FIG. 3 is a negative print of Fig. 2 and represents the view seen under dark-ground illumination.

seen with the naked eye, it becomes more difficult to separate two lines placed 0·2 μ apart. That a gap really exists between these two lines there need be no doubt, for the reader has only to place a magnifying reading glass over the diagram when the two lines will become readily visible. Finally, with the help of the magnifying glass it may also be possible to detect the smallest circle of the series, which measures 0·1 μ in diameter, while two lines situated the same distance apart appear as a single image.

By means of these arguments and the assistance of the illustration we have provided, the reader may be able to convince himself that the limit of visibility is smaller than that of resolution.

We stated that when employing dark-ground illumination both the limits of visibility and of resolution were enhanced. This can be proved by a study of Fig. 3 which consists of a negative prepared from Fig. 2, showing the series of circles and parallel lines precisely as they would appear if seen as brightly illuminated objects against a dark background. From a comparison of Fig. 2 and Fig. 3 it will be observed that the circles and spaces between the parallel lines are more easily visible in Fig. 3 than in Fig. 2. This is so because a stained particle viewed by direct light is always less easy to see than a brightly illuminated object of the same size placed against a dark background. In other words, the contrast between the object and its background is greater in Fig. 3 than in Fig. 2, which results in increased visibility and particularly resolution when using dark-ground illumination. To some extent, therefore, the limit of resolution can be increased by using a more powerful source of

light and increasing the amount of contrast between the object and its surroundings. A good example of a practical illustration showing this point is that of the fixed stars seen on a clear night. Although these can be seen with the naked eye, they are in reality far below the limit of resolution of even the most powerful telescope, and their apparent magnitude, therefore, depends solely on their brightness against the black sky, thus the brighter the star, the larger it appears to be, irrespective of its real size.

Optical Equipment. The choice of suitable equipment[1] is as important as the correct use of it, and strict attention should be paid to the maker's instructions regarding the optimum conditions under which each lens is designed to function. The source of illumination that we have found most suitable for this class of work has been that provided by the short filament low-voltage electric lamp bulb, and a lamp containing a 12-volt 4-amp. bulb should be fitted with a condenser and an iris diaphragm for dark-field and critical bright-field illumination, as well as a ground-glass screen for direct observations.

Before examining a preparation for elementary bodies it is necessary to ensure that the incandescent filament, substage condenser, objective, and eyepiece are accurately centred and in perfect alignment; for with particles of less than 0·3 μ critical illumination is imperative. Stained films should be made on slides of 1·1 mm. thickness, and should be mounted preferably in cedar-wood oil. It is also advantageous to use only preparations which have been previously compressed between two heavy weights for 24 hours, in order to ensure that the coverslip and slide are brought as close as possible to each other. The preparation should be viewed first by direct light with the low-power dry objective to ascertain the types of cells present, and then a 2 mm. oil immersion objective with $\times 10$ and $\times 15$ oculars can be used to search for elementary bodies. Fields showing only scanty elementary bodies should be disregarded and, if possible, clumps of them should be sought. Individual bodies or pairs should be moved into the centre of the field and carefully scrutinized, first with the $\times 10$ and then the $\times 15$ ocular; the fine adjustment should be continuously used and the height of the substage condenser varied in order to focus accurately the light upon individual particles. The substage condenser diaphragm aperture may also be slightly closed in order to give greater depth of focus, and a green filter can be used with added benefit, so as to impart greater contrast to the particle. The length of the microscope draw-tube should also be adjusted to suit the coverslip and slide thickness (see Martin and Johnson, 1931). Each stained film made from pathological material intended to be examined for elementary bodies should be accompanied, if possible, by two control specimens, one from a similar source of normal tissue, and the other from a specimen which is known to contain elementary bodies, for, unless such controls are included in each series, erroneous conclusions are likely to ensue. All three slides should be

[1] The following apparatus has been found useful (van Rooyen, 1937): Patna microscope (Watson) fitted with rackwork draw-tube, universal mechanical stage and centring substage mechanism; compensating eyepieces $\times 10$, $\times 15$, and $\times 20$; apochromatic objectives, 2 mm. ($\frac{1}{12}$ in. oil immersion) of N.A. 1·4 (Leitz) or 2 mm. holoscopic objective of N.A. 1·37 (Watson); 8 mm. dry apochromatic objective of N.A. 0·65 (Zeiss); and 6L dry achromatic objective of N.A. 0·65 (Leitz). The most suitable substage condensers have been found to be either the (Watson) parachromatic dry condenser of N.A. 1 or, better, the holoscopic oil immersion model of N.A. 1·3 or 1·7. With the latter, however, the slides must not exceed 1·3 mm. in thickness (see Bridges, 1936, for details regarding an oil-retaining level for oil immersion substage condensers).

stained together and examined consecutively. The staining reactions and morphology of any virus bodies having been noted, the same field should next be examined by dark-ground illumination. This can be done by revolving the nosepiece bearing the 2 mm. objective away from the slide (which should be firmly fixed to the microscope stage with spring clips), wiping the oil from the surface of the coverslip with a piece of muslin, and then moving either the 6L[1] or 8 mm.[1] lens into position. The objective should next be focused upon the slide, the ground-glass lamp screen

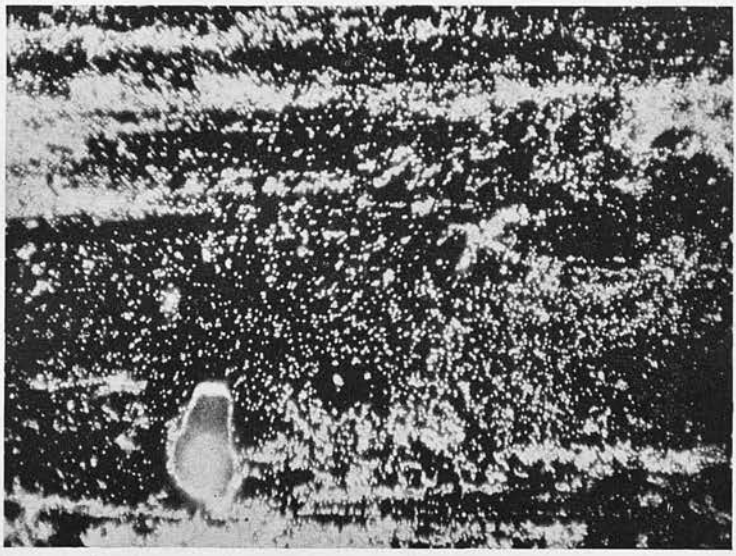


FIG. 4. Photomicrograph taken with the ordinary microscope showing the elementary bodies of vaccinia viewed in the dark-ground field. The picture was taken with a Leitz 6L dry high-power objective and × 14 ocular; the total magnification is × 630.

removed, both iris diaphragms fully opened, and a patch stop or a Travis's expanding stop inserted into position in the condenser substage ring. Dark-ground illumination is now obtainable and it should be possible to determine whether or not the stained elementary bodies previously seen in the field are also refractile to obliquely transmitted light (Fig. 4). By reversing the steps detailed above, it should be possible to restore the direct illumination for the same field. This method enables an observer to study a stained preparation first by direct light and next by dark-ground illumination, without moving the field or having to do any more than insert a Travis's patch stop into the substage condenser.

The Direct Measurement of Elementary Bodies by Micrometry and by Extinction

The measurement of minute microscopical objects presents special difficulties. Three methods are available for the purpose: namely, direct photography, the use of a micrometer eyepiece either of the fixed or vernier screw type, and, thirdly, Nelson's (1909) method of measurement by extinction. None of these methods are perfect, but it may be said that Nelson's principle has many commendable features.

[1] See footnote, p. 4.

Direct Micrometry. Visual and Photographic.

An eyepiece micrometer possessing a scale of 1 cm. divided into 10 mm., and also a stage slide micrometer possessing multiple parallel rulings placed 10 μ (or 0·01 mm.) apart are required.

Method. Set the draw-tube length to the usual working distance. Insert the eyepiece micrometer into the microscope draw-tube, and adjust

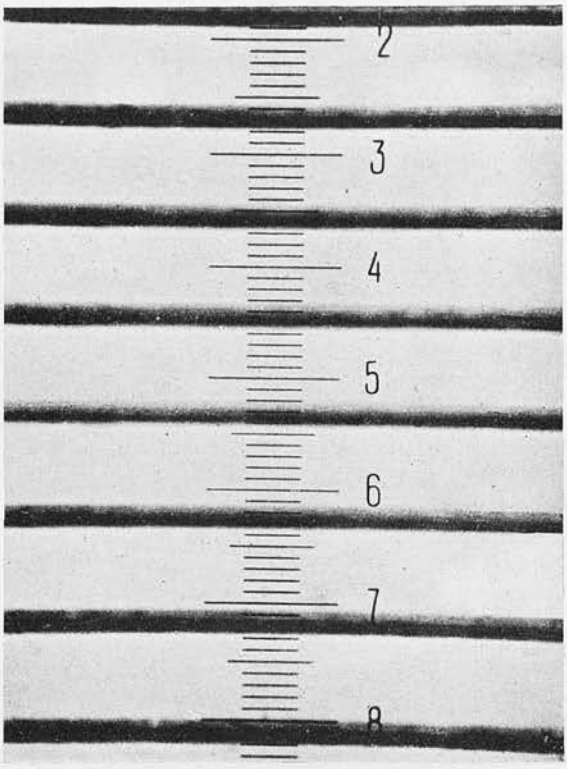


FIG. 5. Shows the stage micrometer and eyepiece micrometer in focus. Each large division on the stage micrometer equals 10 μ, and since 63 divisions on the eyepiece scale equal 70 μ, each small division represents 1·1 μ. By measuring the length of the stage scale with an ordinary ruler the total magnification of the picture can be calculated to be $\times$ 1314·2.

the top lens until the scale is sharply focused. Place the slide micrometer on the microscope stage; place a drop of oil on the scale; focus the divisions carefully with the $\frac{1}{12}$ in. oil immersion objective; count the number of divisions of the eyepiece micrometer which cover those on the stage and ascertain the size of each division on the eyepiece scale.

Example. If 7 divisions on the stage equal 63 small divisions on the eyepiece, then each division on the eyepiece $= \frac{7}{63}$ of a stage division; but since each division on the stage slide equals 10 μ, one space on the eyepiece scale $= \frac{70}{63}$ or 1·1 μ. Having calculated the value of each division of the eyepiece scale for the tube length employed, the stage micrometer should be removed and the stained film of elementary bodies substituted for it. It should then be possible to ascertain approximately what fraction of a space the elementary body occupies, and thus deduce its probable

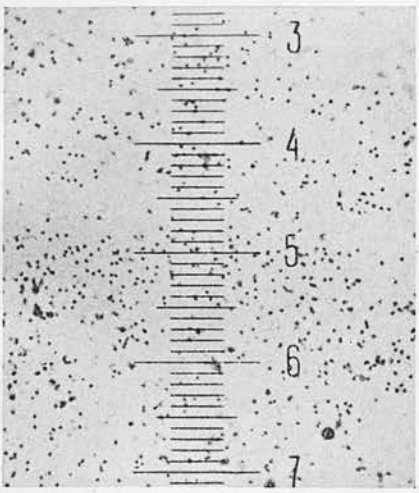


FIG. 6

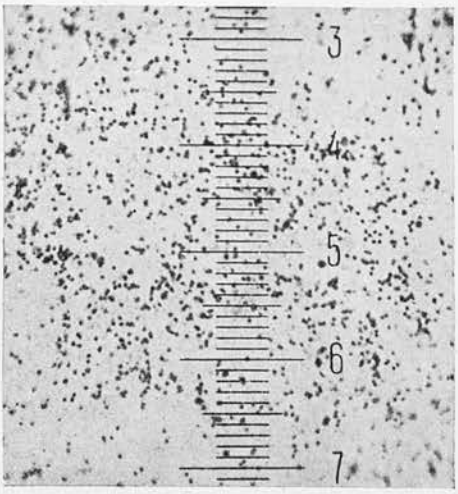


FIG. 7

FIG. 6. Photomicrograph made from a film preparation of vaccinia virus cultivated in the chorio-allantoic membrane of the chick embryo, showing Paschen or elementary bodies.

FIG. 7. Similar picture made from a film of conjunctival secretion obtained from a rabbit infected with myxoma virus, showing elementary bodies.

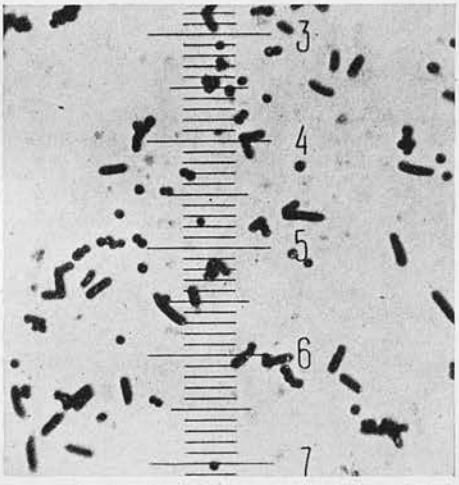


FIG. 8. Film showing a mixed culture of staphylococci and *B. coli*. for comparison with elementary bodies shown above.

All three have been stained by the same method, namely, Paschen's stain for elementary bodies. Each division on the micrometer scale = 1·2 μ. The magnification = ×1,222.

By permission of the *Glasgow Medical Journal* (see vol. **129**, 1).

size. Fig. 5 shows a photomicrograph of the stage micrometer and eye-piece micrometer in focus, and Figs. 6 and 7 show two photomicrographs of the elementary bodies of vaccinia and infectious myxomatosis of the rabbit compared with each other, together with a photograph of staphylococci and *B. coli* stained by the same technique (in Fig. 8). One

small division on the eyepiece scale measures 1·2 μ and each vaccinia elementary body therefore measures about 0·25 μ in size, whereas the myxoma bodies are larger and probably measure 0·3 μ. When making such calculations several bodies in the picture should be viewed with a magnifying glass, measured with a pair of calipers, and the average of 50 or more taken. Each (large) division of the stage micrometer = 0·01 mm. and the total magnification is calculated by measuring these divisions with a ruler. For example, if 7 large divisions of the stage micro-

FIG. 9. Showing an objective designed for the measurement of minute objects by micrometric extinction. It consists of a Leitz 6L dry high-power objective fitted with a flange and graduated iris diaphragm ranging from N.A. 0·65 to N.A. 0·1.

meter equal 92 mm. in length on the projected image on the ground-glass screen of the photographic camera, and each stage division equals 10 μ, then seven divisions equal 70 μ. But 1 mm. equals 1,000 μ and 92 mm. = 92,000 μ so that the total magnification of the illustration is $\frac{92000}{70}$ or 1314·2 times.

Precautions. The following points should be noted when making measurements: (1) The draw-tube length of the microscope must not be altered after calculating the value of the eyepiece micrometer scale. (2) When comparing the relative size of the elementary bodies found in one virus disease with those found in another infection, the preparations studied should be stained by exactly the same method as well as at the same time, with the same set of reagents, before any comparisons are valid. Thus, it is not possible to make any comparative deductions with regard to the size of the elementary bodies present in a film of vaccinia stained by Paschen's method and a film of herpes virus stained by Giemsa's technique, nor is it possible to compare any two specimens which have not been stained under identical conditions. (3) The position of the sub-stage condenser and iris diaphragm must not be altered during the course of the measurements and should be placed wide open at full aperture throughout.

Nelson's Extinction Method.

Nelson's method is probably the most accurate, and yields better results in competent hands than those obtainable either by direct micrometry or photography. A specially constructed objective is required for application of this principle. The writers have used a $\frac{1}{6}$ in. Leitz objective fitted with an internal iris diaphragm attached to a pointer working on a scale upon which graduations in N.A. varying from 0·1 to 0·65 are engraved (Fig. 9). The calibrated values inscribed on the objective were obtained with the use of an apertometer and each figure represents the specified N.A.

Method. In order to measure the size of an elementary body seen by direct white light, the body should be moved into the centre of the field, the objective set at its full aperture of 0·65 N.A. and thereafter gradually reduced in size, by moving the lever controlling the iris diaphragm of the objective, until the elementary body begins to show a hazy and indistinct outline. Only a slight further adjustment is next required to extinguish completely the blurred image of the particle. At this point the N.A. of the objective should be read, and on reference to Table I the approximate size of the body can be calculated.

This method is based on the fact that the size of a particle viewed under the microscope depends, among other factors, on the N.A. of the objective. Thus if the N.A. of the lens be reduced to such an aperture as to bring the object to the point of invisibility, then the correction for anti-point will equal the size of the object.

Example. If a particle seen with white light were found to disappear at N.A. 0·22, then reference to the correction anti-point table will show that it measured 0·521 μ less the difference for 0·02 N.A., which is $2 \times 0{\cdot}0520$, and the object will therefore measure 0·417 μ in size.

TABLE I

	White Light. 45,300 *		*Green Screen.* 50,000 *	
N.A.	μ	*Difference for* 0·1	μ	*Difference for* 0·1
0·1	1·041	..	0·942	..
0·2	0·521	0·0520	0·470	0·0470
0·3	0·348	0·0170	0·315	0·0160
0·4	0·262	0·0090	0·236	0·0080
0·5	0·208	0·0050	0·188	0·0050
0·6	0·173	0·0030	0·157	0·0030
0·7	0·149	0·0020	0·135	0·0020
0·8	0·130	0·0020	0·118	0·0020
0·9	0·116	0·0010	0·105	0·0010
1·0	0·104	0·0010	0·0942	0·0010
1·1	0·0947	0·0009	0·0856	0·0009
1·2	0·0868	0·0008	0·0785	0·0007
1·3	0·0800	0·0007	0·0724	0·0006
1·4	0·0744	0·0006	0·0673	0·0005
1·5	0·0693	0·0005	0·0627	0·0005

* Waves per inch.

THE USE OF THE ULTRA-VIOLET LIGHT MICROSCOPE

The highest magnification attainable with an ordinary microscope fitted with a 2 mm. apochromatic objective of N.A. 1·4 and $\times 20$ compensating eyepiece is about 2,000 diameters, but at so great a magnification there is appreciable loss of detail, and little information can be

derived from the additional size of the image. It is impracticable, therefore, to employ a greater magnification than $\times 1{,}200$ with ordinary light.

The shortest wave-length of ordinary light suitable for visual purposes is 520 mμ and, using a 2 mm. objective with $\times 12$ eyepiece, it is impossible to see a particle less than 0·067 μ in diameter. Due to the work of Kohler (1904), Kohler and von Rohr (1905), and Barnard and Welch (1936 *a*, 1936 *b*), it has been possible to overcome this difficulty by using ultra-violet light for the source of illumination. This possesses a wave-length of 255 mμ, which is about half that of ordinary light, and consequently the resolving power of the microscope becomes doubled. Thus, magnifications of $\times 3{,}200$ can be attained with excellent definition, and accordingly particles even smaller than 0·067 μ can be seen. It is also possible to show greater detail in the structure of larger bacteria, such as *B. megatherium*, than can be demonstrated with the ordinary microscope (see Barnard and Welch, 1936 *b*).

The employment of ultra-violet light necessitates the use of quartz lenses and a special fluorescent eyepiece for preliminary focusing, since the images produced are invisible to the naked eye and have to be recorded on a photographic plate. Furthermore, only unstained objects mounted in glycerine or cane sugar solution on special quartz slides can be examined, since the detail in structure reproduced on the photographic plate depends on varying degress of absorptive power possessed by different specimens, and mountants possessing absorptive properties of their own must be avoided. Great technical difficulties exist with regard to the problem of accurately focusing the objects to be photographed, and these have been overcome by the invention of a specially constructed microscope in which 'back-lash' among the moving components has been reduced to a minimum.

The substage condenser is also of ingenious design, and consists of a high aperture dark-ground illumination condenser with an independent quartz condenser unit mounted centrally within it. Both systems have similar focal lengths, but provision is made for obtaining exact parfocality by altering the relative position of the dark-ground condenser (Barnard and Welch, 1936 *a*).

In order to take a photograph, the object is first brought into focus with ordinary visible light transmitted through an ordinary substage dark-ground condenser fitted to the microscope and, thereafter, by changing the condenser stops and substituting quartz objectives, ultra-violet light is more or less automatically focused upon the specimen and the the image recorded upon a sensitized photographic plate.

The source of illumination recently used by Barnard has been the so-called 'fat' type spark of high intrinsic brilliancy of about 3 mm. in length produced between cadmium and magnesium electrodes. Before reaching the microscope the rays pass through a monochromator, a collecting lens, and two quartz prisms for splitting up the light in order to supply the desired wave-length. Barnard and Welch (1936 *a*) have also employed a mercury vapour lamp yielding a wave-length of 253 mμ at which differential absorption between virus bodies and cell constituents becomes most pronounced.

From the foregoing description it will be observed that, before a suspected elementary body (which must be unstained) can be photographed by ultra-violet light, it must first be located and focused under oblique illumination provided by ordinary light. The use of an ultra-violet light optical system therefore supplies a method by which greater magnification can be applied to unstained objects that are too small to be seen by ordinary dark-ground illumination.

The position is somewhat analogous to that of using a 2 mm. oil immersion objective in order to scrutinize a bacterium which is only just visible when seen through an 8 mm. lens. Although the use of ultra-violet light brings with it the benefits of a higher magnification, it suffers the disadvantage of being applicable only to unstained preparations. Thus, it is impossible to examine a stained film of elementary bodies first with ordinary light and then to photograph it with ultra-violet light, since stained objects cannot be subjected to this method of examination and, consequently, the help and guidance given by tinctorial reactions in a well-stained preparation are lost when working with ultra-violet light. This is not a serious disadvantage where ultra-violet light is used for morphological studies on a large organism such as, for example, *B. megatherium*, but, when dealing with elementary bodies present in pathological exudates which also contain inanimate organic particles of the same size as the virus bodies, staining reactions supply invaluable information. Another drawback associated with this method is the fact that, before any photograph can be taken with ultra-violet light, the operator must first focus the unstained preparation under ordinary light, dark-field illumination, and satisfy himself from what he sees as to whether or not elementary bodies are present in the field, before proceeding to employ the ultra-violet light optical system. In our opinion this introduces a serious source of error, for we are convinced that it is not always possible to distinguish between elementary bodies and cell debris in the ordinary dark-ground field. Certainly, when dealing with highly infective material containing large clumps of elementary bodies such as are sometimes found in vaccinia or ectromelia (Fig. 4), there is usually little room for doubting their character, but when examining material in which the virus bodies are relatively scanty it is often very difficult to differentiate these from protein matter. The position becomes even worse if one attempts to tell from the appearance presented in the dark field alone whether or not virus bodies are present in pathological exudate derived from a disease of unknown aetiology.

The unreliable nature of the results of dark-ground appearances as a test for the presence of virus bodies has been stressed by van Rooyen (1937), who has shown that normal rabbits' conjunctival secretion, when examined by dark-ground illumination alone, may appear to be full of refractile particles that are indistinguishable from elementary bodies. Stained preparations should, therefore, be employed wherever possible, in order to control the appearances seen on oblique illumination. At the present time it may be said that there are no methods which enable us to differentiate with certainty elementary bodies from tissue particles of the same size. Such methods are greatly needed and much work yet remains to be done on this subject.

In summing up, it may be stated that in its design and construction the ultra-violet light microscope reflects the greatest credit on the skill and ingenuity displayed by Barnard and his colleagues, who have been responsible for the present stage of development that the instrument has attained. But the contributions of the instrument to our knowledge of the morphology of viruses have, in our opinion, been relatively small, for it has not yet been able to show any viruses that cannot be demonstrated in an ordinary dark-ground field. Furthermore, in the case of photographs taken at $\times 3{,}200$ diameters magnification with ultra-violet light (see Barnard and Welch, 1936 *b*), the increased detail revealed is insufficient to be of practical value to the morphologist. This automatically raises the question as to whether an elementary body magnified

×1,000 and studied by ordinary dark-ground illumination will show as much detail as it would if it were magnified ×3,200 and reproduced on a photographic plate. This is a debatable point, but we would venture to suggest that a ×3,200 photograph would not reveal as much detail in structure as could be appreciated by the eye if the same object were studied in a dark field under ×1,000 magnification. Another matter worthy of consideration is the fact that when dealing with a minute object such as an elementary body measuring about 0·25 μ in diameter, it is very doubtful if a ×3,200 magnification of such a structure would show any more detail than could be observed in a ×1,000 photographic reproduction. In order to show detail of morphology in objects of the order of size of viruses, we believe that it is necessary to employ a very much higher degree of magnification, and the recently described electron microscope, which permits magnifications to ×20,000 diameters, promises to supply a means whereby this should be possible.

Fluorescence Microscopy

Recently Hagemann (1937) has demonstrated the value of the fluorescence microscope as an aid to the study of virus bodies, which can be made to fluoresce either by directly irradiating them with ultra-violet light or by first treating them with a fluorescent dye such as yellow 2 G.S., a diazo-amino compound of primulin. When examined under the fluorescence microscope the dyed elementary bodies appear as white or bluish-white particles lying upon a darkly coloured background. The method has also been used to examine elementary bodies within living cells.

Sources of Ultra-violet Light.

Barnard and Welch (1936) state that the best fluorescent effects are exhibited by organisms viewed under dark-ground illumination and consequently an intense source of ultra-violet light must be used in order to get the best results. The carbon or metallic arc may be used, but this suffers from the disadvantage of possessing too low an ultra-violet light output in relation to the total light emitted.

The mercury vapour lamp is also useful but, although its ultra-violet light output is high, its intrinsic brilliancy is low, since the rays are dispersed over too wide an area, and consequently it is unsuitable for microscopy. Both the above described varieties of illuminant are also unsatisfactory because they necessitate the use of a filter in order to absorb the visible rays and there is consequently much loss of brilliancy in the dark-ground field.

The high-tension electric spark discharge—this is produced between magnesium electrodes and, according to Barnard and Welch (1936), represents the best source of ultra-violet light obtainable. On account of its high intrinsic brilliancy this form of illuminant is not only available for fluorescence microscopy but also for direct ultra-violet light photomicrography.

Optical equipment. The following components are required: an ultra-violet light lamp, a quartz or crown glass collecting lens, and a suitable filter. The microscope should be fitted with a quartz dark-ground substage illuminating condenser and the ordinary glass reflecting mirror replaced by a polished metal one. Fluorescence may be observed visually through ordinary apochromatic objectives with compensating eyepieces, but if photographs with ultra-violet light be required, then quartz objectives must be employed.

Material for examination should be spread on quartz slides, treated with primulin (*vide infra*), mounted in Ringer's solution, physiological saline, or distilled water, and covered with a type of cover-glass that is opaque to ultra-violet rays.

Himmelweit (1937) has used vertical illumination[1] for his experiments on fluorescence microscopy and has studied the multiplication of vaccinia virus in the chorio-allantoic membrane of the developing chicken embryo.

Procedure.

1. Material containing the virus is spread on a quartz glass slide.
2. It may be fixed, if required, with 96 per cent. alcohol or 1 to 4 per cent. formalin for 5 to 10 minutes before staining.
3. Treat with a 0·1 per cent. solution of primulin containing 0·2 per cent. phenol in distilled water for 15 seconds.
4. Wash with distilled water, dry, and examine, suitably mounted, under the fluorescence microscope.

When using living tissues phenol should be omitted from the 'fluorochrome' solution. Primulin is the most effective dye for this class of work and it dissolves in water to produce a bluish fluorescence, which is transferred to organisms impregnated with it.

Hagemann (1937) states that the use of the dye makes it possible to detect certain morphological details that would otherwise be unrecognizable in canary-pox, vaccinia, and ectromelia viruses. He also recommends the principle for the morphological study of bacteria and trypanosomes. For other work on fluorescence microscopy see Kufferath (1935), Sutro (1936), and Haitinger (1938).

THE USE OF THE MAGNETIC ELECTRON MICROSCOPE

The magnetic electron microscope which has also been named the 'super microscope' has been developed from the work of Marton (1934, 1937), Martin, Whelpton, and Parnum (1937), and others. Recently, von Borries, Ruska, and Ruska (1938) have improved the instrument further, and have succeded in obtaining photographs of bacteria and viruses magnified $\times 20{,}000$ times with reasonably good definition. The principle on which the instrument operates rests on the fact that free electrons and beams of electrons can be deflected by an electro-magnetic field. In order to use this principle for optical purposes, the magnetic fields have been applied in such a way that they act on the electron beam in the same manner as optical lenses do on beams of light. Such a field or 'magnetic lens' can be produced by a current-carrying coil, as shown in Fig. 10. The coil is enclosed in an iron casing which only leaves a narrow annular slit at 90° to the axial point where the maximum concentration of field is required. When the current flows through the coil, the free ends of the iron casing are transformed into magnetic poles, and between them a magnetic field develops possessing the properties of an almost faultless convex lens, and the path of a ray of electrons through such a lens is illustrated in Fig. 10. It is, however, complicated by the fact that the electrons, while passing through the coil, are not only deflected from their original direction in the plane of the drawing, but also spirally around the axis of the coil.

In every single point of the curve it is only the magnetic component perpendicular to the momentary direction of the ray which acts on it, and produces a force at right angles to both these directions. Thus a

[1] Leitz Ultropak outfit.

curve originates which, from point to point, changes its direction in space and lies on the surface of a spindle possessing a curved axis.

Such a magnetic lens has no definite focal distance, but can be varied by altering the coil current which in turn alters the force of the magnetic field and, consequently, the focal length of the lens. As the focal length varies, the electron-optical image not only becomes more or less sharp, but, over and above that, turns on itself, i.e. round the optical axis, in accordance with the spiral ray deflection described.

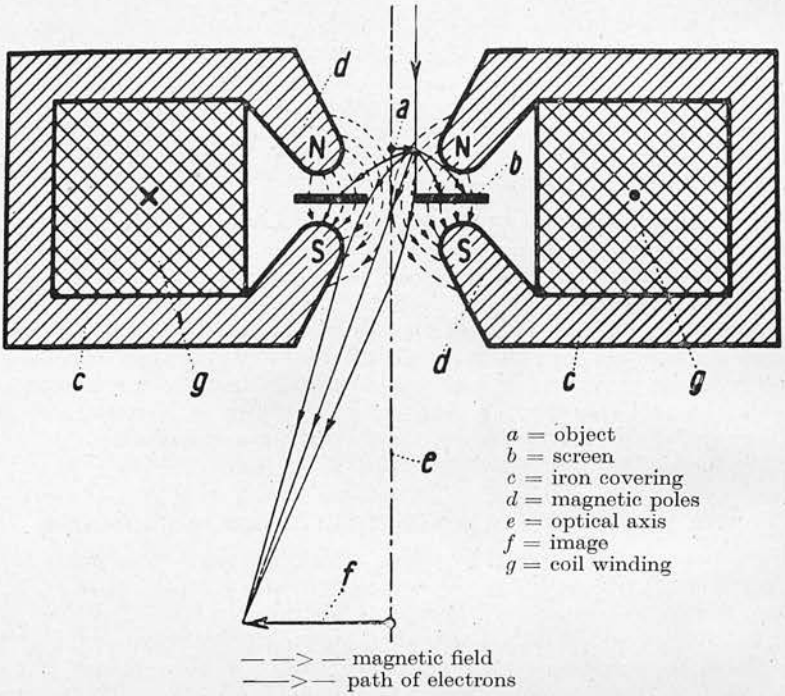


FIG. 10. Diagram showing the path of electron rays through an electro-magnetic lens, illustrating the principle on which the 'super microscope' constructed by Borries *et al.* (1938) is designed. Reproduced by permission of the *Klinische Wochenschrift* (see vol. **17**, 921).

Since electron beams can only exist in a vacuum, any object which is to be inspected by an electron-optical system must itself be placed within the vacuum, and since electron beams are not directly visible to the eye, the image formed must be made visible either by a luminescent screen or recorded on a photographic plate.

The objects photographed by von Borries *et al.* have been placed on collodion foils 20 μ thick and observed in 'transmitted light'. The varying depth of shadows seen in different objects is brought about by the fact that the electron beams which fall on the object are almost parallel, and are scattered to a different extent, varying in proportion to the 'mass thickness' (i.e. density $\times$ irradiated thickness) of the object. Thus the greater the mass thickness, the greater the scattering and the darker the picture. The more widely scattered rays are eliminated by not allowing them to pass through the minute aperture of the objective lens (see Fig. 11). Parts of the object having a greater mass thickness therefore appear darker than those having a lesser mass thickness. In

this manner, the electron-optical picture can reveal the mass distribution of different minute objects. The instrument employed by von Borries *et al.* is constructed as follows: The source of light is situated at the top of the instrument and the ocular is placed at the bottom of it. The

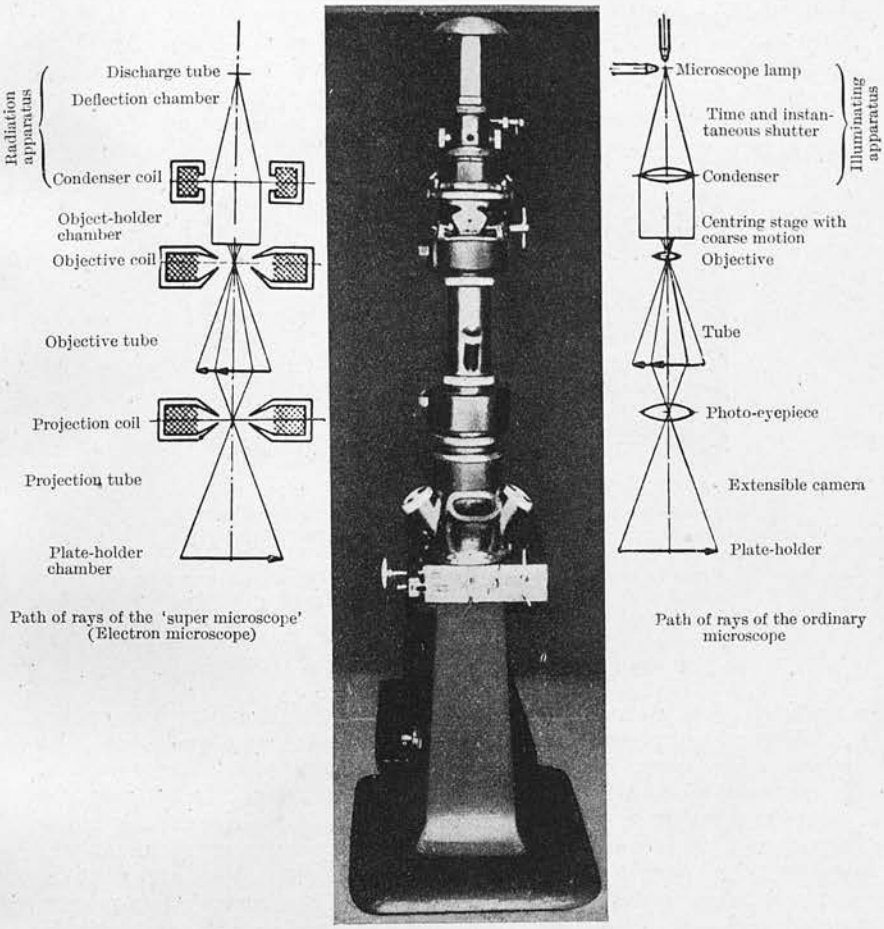


FIG. 11. The Magnetic Electron Microscope. *On the left* is a diagram showing the path of the rays through the electron microscope. *Centre*, a photograph of the microscope. *On the right*, glass lenses have been substituted for the electro-magnetic lenses depicted in the drawing on the left. (After von Borries *et al.*, 1938.) Reproduced by permission of the *Klinische Wochenschrift* (see vol. **17**, 921).

cathode which carries the high tension lies above the head of the observer and the beam falls downwards perpendicularly from the cathode, inside a metal cylinder containing a vacuum, to reach finally a luminescent screen or photographic plate. The electron beam is first collected by an electromagnetic lens which corresponds to a microscope condenser and it next illuminates the object. The 'objective lens' projects a real image of the object in first stage magnification, which can, if required, be visually inspected by insertion of an 'intermediate picture screen'. Part of the intermediate image passes through a hole in the intermediate picture screen and is then magnified a second time by a 'projector lens' which

appears as the final image on the lower (second) luminescent screen or photographic plate. The middle part of Fig. 11, shows the general construction of the 'super microscope'. At the upper end there is a porcelain tube which isolates the incandescent or cold cathode from the rest of the instrument which is earthed. Below there is a blocking chamber by which the rays can be deflected by an electro-magnetic field so that the object can be protected from unnecessary irradiation. Below the blocking chamber there is a condenser coil which can be moved all round, so that the beam can be exactly focused on the object. Through the next component or 'object lock' the specimen to be examined can be inserted into the microscope without having to disturb the vacuum. The

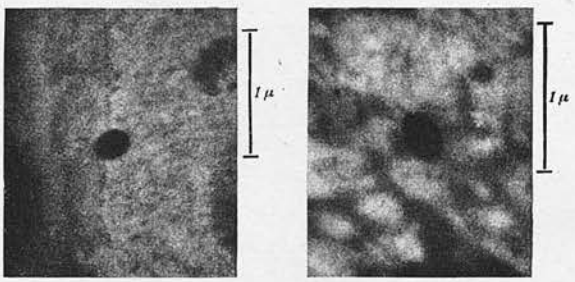


FIG. 12. Photographs taken with the super electron microscope under × 19,000 and × 22,000 magnification by von Borries *et al.* (1938), showing the elementary bodies of infectious myxomatosis of the rabbit. Note the oval shape of these structures and compare this illustration with Fig. 7, showing the appearance of the same variety of elementary bodies when photographed with the ordinary microscope under × 1,222. Reproduced by permission of the *Klinische Wochenschrift* (see vol. **17**, 921).

object can now, if required, be moved perpendicularly to the optical axis during inspection in an analogous manner to the adjustment of an ordinary microscope mechanical stage, so that any desired part of the preparation can be projected on the viewing screen. In addition, the object is movable in the axis of the instrument which corresponds to the action of the coarse adjustment of an ordinary microscope. Below the removable top part of the instrument is the objective lens, and next the tube with lateral openings for the inspection of the intermediate picture, through which the whole preparation can be viewed so that any particular part of the field once seen can be relocated. Below the intermediate picture device the projector lens and the lower tube (projector tube) are mounted, and through the wide opening of the latter the final picture can be inspected. At the back of the instrument a wide tube leads from the projector tube to a high vacuum pump. At its lower end the microscope is closed by a lock through which—again without disturbing the vacuum—a camera can be inserted, opened in the vacuum, and exchanged for a luminescent viewing screen. The current for the coil lenses and the high-tension exciter is controlled from a switchboard during the course of the microscopical examinations. For additional information regarding the design and construction of the electron microscope the reader is referred to the work of Marton (1934 *a*, 1935, 1936, and 1936 *a*).

With this instrument von Borries *et al.* state that structures of 10 mμ can be distinctly recognized. They have been able to see the internal structure of bacteria, and claim that *Bacillus coli* is composed of an

ectoplasm and an endoplasm, also that the elementary bodies of myxoma are not circular in shape as they appear under the ordinary microscope, but are in reality oval (Fig. 12). They suggest that such evidence might in the future make it possible to identify elementary bodies from their morphological appearances alone.

REFERENCES

AIRY, G. B. (1834), *Camb. Phil. Trans.*, **5**, 283.
BARNARD, J. E., and WELCH, F. V. (1936), *J. R. micr. Soc.*, **56**, 361.
—— —— (1936 *a*), *J. R. micr. Soc.*, **56**, 365.
—— —— (1936 *b*), *Practical Photo-micrography*, p. 333, Arnold, London.
BRIDGES, C. B. (1936), *Science*, **84**, 335.
COLES, A. C. (1929), *Brit. med. J.*, **2**, 91.
HAGEMANN, P. K. H. (1937), *Münch. med. Wschr.*, **84**, 761.
HAITINGER, M. (1938), *Fluorescenz-Mikroskopie*, Akademische Verlagsgesellschaft, m.B.H. Leipzig.
HIMMELWEIT, F. (1937), *Lancet*, **2**, 444.
JOHNSON, B. K. (1928), *J. R. micr. Soc.*, **48**, 144.
KÖHLER, A. (1904), *Z. wiss. Mikr.*, **3**, 129, 273.
KÖHLER, A., and VON ROHR, M. (1905), *J. R. micr. Soc.*, **25**, 513.
KUFFERATH, A. (1935), *Fortschr. Med.*, **53**, 306.
MACKIE, T. J., and MCCARTNEY, J. E. (1938), *A Handbook of Practical Bacteriology*, Livingstone, Edinburgh.
MARTIN, L. C. (1925–6), *Trans. Opt. Soc., Lond.*, **27**, no. 4.
MARTIN, L. C., and JOHNSON, B. K. (1931), *Practical Microscopy*, Blackie, London.
MARTIN, L. C., WHELPTON, R.V., and PARNUM, O. H. (1937), *J. sci. Instrum.*, **14**, 14.
MARTON, L. (1934), *Bull. Acad. Belg. Cl. Sci.*, **20**, 430.
—— (1934 *a*), *Phys. Rev.*, **46**, 527.
—— (1935), *Bull. Acad. Belg. Cl. Sci.*, **21**, 553, 606.
—— (1936), *Extrait de la Revue de Microbiologie appliquée à l'Agriculture, à l'Hygiène, à l'Industrie*, no. 3, mai–juin, Baillière, Paris.
—— (1936 *a*), *Bull Acad. Belg. Cl. Sci.*, **22**, 1336.
—— (1937), *Bull. Acad. Belg. Cl. Sci.*, **23**, 672.
MERLING-EISENBERG, K. B. (1938), *Brit. J. exp. Path.*, **19**, 338.
NELSON, E. M. (1909), *J. R. micr. Soc.*, **29**, 549.
SUTRO, C. J. (1936), *Arch. Path. Lab. Med.*, **22**, 109.
VAN ROOYEN, C. E. (1937), *Zbl. Bakt.*, Abt. I, orig., **139**, 130.
VON BORRIES, B., RUSKA, E., and RUSKA, H. (1938), *Klin. Wschr.*, **17**, 921.

CHAPTER II

THE STAINING OF ELEMENTARY BODIES

Glass Slides

THE variety found most suitable for preparing films of elementary bodies are those made of crystal glass varying from 1 to 1·1 mm. in thickness (Coles, 1935). This size suits the working distance of most microscope substage condensers, and consequently slides less than 1 mm. or more than 1·3 mm. in thickness must not be used unless a specially designed condenser is employed.

It is essential that glass slides should be thoroughly cleaned before attempting to make films of material to be stained for elementary bodies, and the following two methods have been recommended: (1) Place the slides in chromic acid for 24 hours, wash with running water for 24 hours, and store in 50 per cent. alcohol until required, after which they should be wiped and dried with clean gauze. (2) Slides may also be cleaned by boiling them for 15 to 30 minutes in a solution consisting of concentrated sulphuric acid 6 c.c., potassium bichromate 6 gm., and water 100 c.c., the liquid being periodically agitated with a glass rod to prevent adhesion of the slides. After removal from the bichromate solution, they should be thoroughly washed in running water for several hours, and then stored in 50 per cent. spirit in an air-tight container.

METHODS FOR THE STAINING OF ELEMENTARY BODIES

The elementary bodies of vaccinia were first demonstrated in stained film preparations made from vesicle fluid by Dr. John Buist working at the University of Edinburgh in the year 1886. Buist's observations are of great historical interest, for although he recognized the aetiological significance of the minute bodies he found in vesicle fluid, and was undoubtedly the first observer to have noticed them, it was not until Gordon (1937) and Mackie and van Rooyen (1937) drew attention to his work that his claims were recognized.

In a later publication Buist (1887) clearly revealed from an illustration that he not only differentiated these small objects from cocci, but also appreciated the difficulty of staining them with simple aqueous dyes. As a matter of interest, we ourselves recently repeated the staining technique originally practised by Buist over fifty years ago, and found that the results it yielded compared very favourably with modern methods.

The following is a list of different staining methods which have been found suitable for the demonstration of elementary bodies:

Buist's (1887) Method

This is mainly of historical interest, and is carried out as follows:

Solution required.

Ehrlich-Koch solution: consisting of saturated alcoholic solution of methyl violet, 5 to 20 c.c., and 100 c.c. of aniline water consisting of 2 per cent. aniline shaken up with 98 c.c. of water and filtered.

To demonstrate elementary bodies in pathological material it is important to employ the most suitable staining method. The affinity of virus bodies for different dyes varies considerably. For instance, Paschen's stain is excellent for demonstrating the viruses of variola, vaccinia, herpes, ectromelia, fowl-pox, myxoma and others, but it fails to reveal the agents of psittacosis, lymphogranuloma inguinale and inclusion conjunctivitis. Likewise, Castaneda's stain appears to yield the best results with psittacosis alone; whilst on the other hand, dilute Giemsa solution gives excellent coloration effects and can be used for demonstrating the majority of viruses. Unfortunately, the latter suffers from the disadvantage that it tends to precipitate too readily and is liable to produce artefacts.

According to my own tests with the elementary bodies of fowl-pox, ectromelia, and vaccinia, they appear to be Gram-negative and non-acid-fast, but it would be unwise to draw any conclusions from such limited data until a systematic study of the staining properties of viruses has been conducted. This aspect of the subject is of great importance for the discovery of staining methods which would enable us to differentiate virus bodies from tissue debris, and would be of fundamental value. Not only would it be of help in the study of the known viruses, but it would assist in diminishing the number of bad mistakes which/

which have been made from time to time by a few workers who have been seriously misled by the appearances of tissue artefacts.

The ensuing text contains an account of staining methods which I have tested myself and found effective for the purposes specified.

Procedure.

1. Cover-glass preparations are made directly from typical vaccine vesicles, and fixed by passing them three times through the flame of a Bunsen burner.
2. Stain for 12 hours in freshly prepared aniline methyl violet solution.
3. If quicker staining is required, then the solution must be gently heated for half to one hour.
4. Wash in distilled water.
5. Next treat in 60 per cent. alcohol.
6. Dehydrate in absolute alcohol.
7. Mount immediately in balsam and benzol.
8. Contrast staining is usually unnecessary, but if desired, a solution of vesuvin (Bismarck brown) or chrysoidin may be used after washing in 60 per cent. alcohol.

Paschen's (1906, 1913) Method (see also van Rooyen, 1937)

This method was employed by Paschen for staining the elementary bodies of vaccinia, variola, herpes, varicella, ectromelia, and Shope's fibroma virus, and we have found it to be among the best and most reliable methods for the staining of elementary bodies.

Solutions required.

Saturated alcoholic solution of basic fuchsin, 10 c.c.; 5 per cent. solution of carbolic acid, 90 c.c. Alternatively the stain could be made up in two parts: Solution (A) consisting of basic fuchsin, 0·3 gm., ethyl alcohol, 10 c.c.; and Solution (B) containing phenol, 5 gm., and distilled water, 95 c.c. Solutions (A) and (B) are mixed before use.

Löffler's flagellar mordant. 20 per cent. aqueous solution of tannic acid, 100 c.c.; ferrous sulphate (saturated solution), 50 c.c.; basic fuchsin (saturated alcoholic solution), 10 c.c. (see von Prowazek, 1912).

Procedure.

1. Make film on slide.
2. Place in distilled water for 3 minutes.
3. Dry in air.
4. Fix in absolute alcohol for 5 minutes.
5. Dry in air.
6. Treat with Löffler's mordant and heat gently until steam rises, and allow it to act for 10 minutes.
7. Wash thoroughly in water.
8. Stain with carbol fuchsin solution, gently heating the stain until steam rises for 1 minute, and allow it to remain for a further 10 minutes.
9. Wash rapidly in distilled water and dry with blotting-paper.
10. Mount in neutral mountant or cedar-wood oil.

Elementary bodies appear deep red in colour when stained by Paschen's method.

The Method of Staining Viruses by Giemsa's (1910) Dilute Solution

This stain has been used by many pioneer workers in their researches on virus diseases. Almost every type of elementary body can be stained by this technique, and in skilled hands very beautiful tinctorial effects can be obtained. The stain possesses the advantage of staining red and white corpuscles simultaneously in blood films, but unfortunately it

suffers from the drawback of being liable to deposit too readily when standing over a period of time, and moreover different samples of the dye may vary considerably in their staining affinities for elementary bodies (see Craigie, 1933). The pH of the tap water used for diluting the stain may also influence the results, if it is unduly acid or alkaline in reaction.

Coles's (1935) Method

This is probably one of the best methods of applying Giemsa's stain and has been successfully employed to demonstrate the elementary bodies of vaccinia, herpes, psittacosis, and other virus diseases.

Apparatus needed. A curved glass plate 10 in. long and 4 in. broad, possessing a concave upper surface of about 18 in. in radius of curvature. A flat tin box 12 in. long, 6½ in. broad, and 1 in. deep, fitted with an air-tight lid.

Solutions required.

Stain. Giemsa's powder (Grübler), 0·75 gm., glycerine, 25 c.c., and industrial methylated spirit, 75 c.c., are thoroughly mixed and well shaken.

Differentiating solution. Orange G, 1 gm.; tannic acid, 5 gm.; and water, 100 c.c.

Procedure.

1. Make thin films as usual.
2. Fix by placing over the mouth of a wide-necked jar—containing crystals of iodine—until the film turns light brown after about ½ to 1 minute exposure (see Plimmer, 1913).
3. Treat with absolute alcohol for 5 minutes.
4. To stain, place the film face downwards on the curved plate and run underneath it three drops of the stock Giemsa's solution and three drops of methylated spirit to which is added three times as much tap water.
5. Place the curved plate in the air-tight metal box and allow the stain to act for 24 hours.
6. Renew the stain at the end of the first day and allow it to act for a further 24 hours.
7. Wash the film in tap water, and differentiate it if necessary by placing a drop of the differentiating solution on one end of the film, and allowing it to act for a few moments before washing off with water.
8. Dry the film with blotting-paper, and mount in neutral mounting medium.

Hosokawa's Modification of Giemsa's Staining Method
(see Taniguchi *et al.*, 1934 *a*)

The elementary bodies of molluscum contagiosum, smallpox, varicella, herpes, zoster, fowl-pox, and rabbit myxomatosis may all be stained by this technique irrespective of whether they occur intracellularly or extracellularly. The stained virus bodies appear reddish-purple or bluish-purple. We have noticed that Hosokawa's solution has a tendency to overstain the granules of eosinophil polymorphs, and unless the results are carefully controlled, mistakes are liable to occur.

Solutions required.

Fixative. Pure methyl alcohol, 100 c.c.; formalin, 5 c.c.; and glacial acetic acid, 1 c.c.

Eosin solution. 1 per cent. watery eosin solution (Grübler B.A.).

Stain. Eosin methylene blue, 4 gm.; azur I, 0·8 gm.; crystal violet, 0·05 gm.; these are dissolved in a mixture of methyl alcohol, 250 c.c., and glycerine, 250 c.c. This stock solution is diluted before use (*vide infra*).

Procedure.

1. Films are made on glass slides in the ordinary manner, and allowed to dry in air.
2. Treat with fixative for 2 minutes; wash in water.
3. Flood with eosin solution and heat over a flame until steam rises for half a minute; then wash in water.
4. Hosokawa's stain is next applied by adding 1 c.c. of stock solution to 50 c.c. of distilled water, and applying the diluted mixture to the slide for 30 to 40 minutes at room temperature.
5. The stain is washed off with water, the slide is dried in air and examined under the microscope.

The Taniguchi *et al.* (1934 b) Modification of Giemsa's Method for Staining Elementary Bodies

This method has proved useful for staining the elementary bodies of variola, vaccinia and varicella viruses as well as certain types of cell inclusion.

Solutions required.

Fixative. Pure methyl alcohol, 100 c.c.; formalin, 5 c.c.; glacial acetic acid, 1 c.c.

Stain. 1 per cent. watery eosin-Giemsa solution (Grübler B.A.).

Procedure.

1. Make smears and dry thoroughly in air.
2. Fix for 2 minutes in fixative solution.
3. Wash in water.
4. Flood with eosin-Giemsa staining solution and gently warm over flame for ½ minute if staining vaccinia or variola virus, and for 1 minute if films of chickenpox or herpes virus are being treated.
5. Wash with water, dry, and examine. Elementary bodies stain bluish-purple.

Craigie's (1933) Method

Solutions required.

(*a*) A 2 per cent. aqueous solution of mercurochrome.

(*b*) The stain employed has the following composition: 2 per cent. aqueous solution mercurochrome, 220 Soluble (H.W. & D.), 1 c.c.; 7 per cent. aqueous solution M/5 Na_2HPO_4, $2H_2O$ (or Na_2HPO_4, $12H_2O$), 5 c.c.; 1 per cent. aqueous solution methylene azure A (azur I) (certification No. NAz4), 1 c.c.; 1 per cent. aqueous solution methylene blue (U.S.P. med.) (certification No. NA5), 25 c.c.; and distilled water, 75 c.c.

The constituents are added in the order stated and the mixture, which should not be filtered, retains its staining qualities unimpaired at room temperature for three months.

Procedure.

1. Spread films very thinly as in preparing a blood film when working with preparations derived from the skin; tissue scrapings should be suspended in distilled water before films are made.

2. Wash in two changes of distilled water for 5 minutes; dry.
3. Fix in methyl alcohol for 5 to 10 minutes; dry.
4. Rinse in distilled water and shake or blot off excess water. Cover film with 6 drops of 2 per cent. aqueous mercurochrome for 5 to 10 minutes. Rinse rapidly in tap water and later in distilled water; blot off excess water.
5. Cover film with stain (6 drops) for 5 to 10 minutes.
6. Rinse off stain as rapidly as possible and blot and dry immediately or, alternatively, simply blot and dry without rinsing. The only part of the technique which requires care is the final step of removing the stain and drying the film, since water, if left in contact with the stained film, tends to dissolve out the dye from the elementary bodies. Hence it is necessary to dry the preparation as quickly as possible. Elementary bodies stain bluish-purple.

Herzberg's (1934) Victoria Blue Stain (Ordinary Method)

Victoria blue was first shown by von Prowazek (in 1905) to be a suitable dye for the staining of elementary bodies of vaccinia. With it the elementary bodies of vaccinia, varicella, herpes, and canary-pox have been demonstrated in smears prepared from infective tissue. Herzberg (1936) has also been successful in detecting the individual differences in size exhibited by these viruses when compared with one another under the microscope. When stained by this method the individual elementary bodies appear dark blue in colour and the dye shows little affinity for staining the cell cytoplasm.

Solution required.

The *stain* is prepared as follows: 9 gm. of Victoria blue 4 R. (Bayer) are dissolved in 300 c.c. of distilled water, heated to 60° C. for 30 minutes, placed in a brown-coloured bottle for 14 days, and filtered before use.

Procedure.

The procedure employed for staining elementary bodies of different virus diseases varies slightly and may be summarized as follows:

Vaccinia. Films are made from infected corneal tissue 3 to 4 days after infection, dried in air for 24 hours, placed in distilled water for 10 minutes, dried at 37° C. for 1 hour, stained with 3 per cent. Victoria blue solution for 5 minutes, rinsed with distilled water, dried, and examined. (See also Haagen and Kodama, 1934.)

Varicella. Thin films are made from vesicle fluid, dried in air for 24 hours, stained with Victoria blue solution for 20 minutes, washed with distilled water, and examined under the microscope.

Herpes. Impression preparations are made from infected rabbit cornea, dried for 24 hours in air, placed vertically in a jar of distilled water for 10 minutes, dried at 37° C. for 1 hour, stained with Victoria blue for 30 minutes, rinsed in water, dried, and examined.

Ectromelia. Films are made from the feet of infected mice, dried for 24 hours, stained for 10 to 30 minutes, rinsed in distilled water, dried, and examined.

Canary-pox. Films are made from infected tissue, stained for 5 minutes, washed in distilled water for 30 seconds using two changes until clear, dried, and examined.

Herzberg's (1934) Victoria Blue Contrast Staining Method

This has been used for demonstrating the elementary bodies of vaccinia and canary-pox, and is carried out as follows:

Solutions required.

(*a*) *Nuclear red.*[1] 5 gm. of aluminium hydroxide are melted in 100 c.c. of hot distilled water, filtered through paper, 0·1 gm. of nuclear red added, dissolved by boiling, again filtered, and 0·5 c.c. of a 10 per cent. solution of acetic acid added.

(*b*) *Light green.*[1] 1 gm. of the dye is dissolved in 100 c.c. of distilled water, and 0·5 c.c. of 10 per cent. acetic acid solution is then added.

(*c*) *Victoria blue.* A 0·1 per cent. solution of Victoria blue 4 R. solution is prepared by diluting the 3 per cent. stock stain already described; to this is added 0·5 c.c. of 10 per cent. acetic acid.

Procedure.

1. Films are prepared as usual.
2. Place in distilled water for 10 minutes.
3. Dry in air for 1 hour.
4. Treat with nuclear red solution for 15 minutes.
5. Rinse for 30 seconds in distilled water using two changes.
6. Dry in air for 30 minutes.
7. Treat with 1 per cent. light green solution for 2 minutes.
8. Wash with distilled water for 5 seconds, twice.
9. Treat with 10 per cent. tartaric acid for 3 minutes.
10. Wash twice in distilled water for 5 seconds each time.
11. Stain with 0·1 per cent. Victoria blue solution for 20 seconds.
12. Wash twice in distilled water as before.
13. Dry in air and examine under the microscope.

Gutstein's (1937) Methyl Violet Stain

This stain has been used for staining vaccinial and other elementary bodies.

Solutions required.

(*a*) 1 per cent. methyl violet in distilled water.

(*b*) 2 per cent. sodium carbonate.

Procedure.

Clean slides in nitric acid, and rinse with alcohol-ether.

1. Prepare films, dry in air or incubator. If much protein is present rinse first in saline, and then in distilled water.
2. Fix film in methyl alcohol for half an hour or more, and place the slide in a dry Petri dish.
3. Mix equal parts of solutions (*a*) and (*b*) in a test-tube, filter immediately on to slide, cover the dish with a lid, and incubate it at 37° C. for 20 to 30 minutes.
4. Rinse in distilled water.
5. Dry in air, mount in cedar-wood oil or liquid paraffin. When seen under the microscope, elementary bodies stain light violet.

The above staining procedure can be shortened by fixing the film by gentle flaming, pouring the filtered mixture of (*a*) and (*b*) on the slide, and slowly heating it over a Bunsen burner until steam rises. After 3 to 5 minutes, the slide should be washed with water, dried, mounted, and examined in the usual way.

[1] Hollborn (Leipzig) standard stains.

Gutstein's (1937) Modification of Herzberg's Victoria Blue Stain

Solutions required.

(*a*) Victoria blue 4R., 1 gm.; alcohol, 10 c.c.; and distilled water, 90 c.c.
(*b*) 0·02 per cent. potassium hydroxide in water.

Procedure.

1. Invert the film and place the slide face downwards on two supporting pieces of capillary tubing placed in a petri dish.
2. Mix equal parts of solutions (*a*) and (*b*) together, filter at once, and run under slide.
3. Cover the petri dish with a lid and allow it to stand overnight at room temperature.
4. Remove and rinse the slide with distilled water.
5. Dry and mount in neutral balsam or paraffin. In a well-stained preparation the elementary bodies should stain dark blue.

Castaneda's Method (see Zinsser and Bayne-Jones, 1934; also Castaneda, 1930)

This stain is excellent for demonstrating rickettsiae in films of infective tissues, and Bedson (1937) has recommended it for staining psittacosis virus bodies in the spleen of experimentally infected mice.

Solutions required.

Buffer. Potassium dihydrogen phosphate (KH_2PO_4), 1 gm., is dissolved in distilled water, 100 c.c., and added to sodium phosphate (Na_2HPO_4, $12H_2O$), 25 gm., dissolved in distilled water, 900 c.c. The mixture is adjusted to pH 7·5, and 1 c.c. of formalin is added as a preservative.

Stain. Methylene blue, 1 gm.; methyl alcohol, 100 c.c.

Counterstain. 0·2 per cent. safranine O[1] in distilled water, 1 part; and 0·1 per cent. acetic acid, 3 parts.

Procedure.

1. Spread very thin films of the material on clean glass slides.
2. Stain for 3 minutes with a mixture consisting of 20 c.c. of buffer solution; 1 c.c. of formalin; and 0·15 c.c. of stain.
3. Pour the stain off the slide without washing.
4. Treat with counterstain for 1 to 4 seconds (never more than 5 seconds).
5. Wash with tap water, dry, and examine. Elementary bodies stain deep blue.

Bedson and Bland's Modification of Castaneda's Stain (see Bedson, 1937)

This method has been employed for staining psittacosis virus particles.

Solutions required.

Stain. This is a mixture composed of phosphate buffer solution (pH 7·0), 9·5 c.c.; neutral formalin, 5 c.c.; and borrel blue, 10 c.c.

Counterstain. A 1 per cent. aqueous solution of safranine is employed.

Procedure.

1. Smears or impression preparations are made on clean slides.
2. Fix with methyl alcohol.
3. Treat for 2 minutes with staining solution.
4. Wash with tap water.
5. Counterstain for a few seconds, and dry with blotting-paper.

[1] National Aniline and Chemical Co., New York.

Lépine's modification of Castaneda's Method

Solutions required.

Stain. A stock solution of stain is prepared by dissolving azur II (Grübler or Gurr) to make a 1 per cent. solution in 0·5 per cent. phenol in water; this is diluted before use by adding 10 drops of the solution to a second mixture consisting of 10 c.c. of distilled water, 10 drops of neutral formalin, and 5 drops of 1 per cent. potassium carbonate. This mixture is heated before being applied to the film.

Counterstain. This consists of safranine diluted 1 in 2,000 in distilled water.

Procedure.

1. Films are made and fixed after the manner described above.
2. Stain with hot solution for 5 to 10 minutes.
3. Wash in tap water.
4. Counterstain with safranine for 5 to 10 seconds.
5. Wash in tap water and dry with blotting-paper.

Bedson (1937) has particularly recommended this method, which stains psittacosis E.B.'s deep blue.

Six Methods described by Taniguchi *et al.* (1932) for Staining the Elementary Bodies of Variola

Method one.

Solutions required.

(*a*) Pure acetone (Merck).

(*b*) *Mordant.* Add 1 per cent. cadmium iodide to neutral concentrated liquid formalin; the yellow precipitate which is formed is next redissolved by adding the minimum quantity of concentrated hydrochloric acid.

(*c*) Eosin, 0·2 gm., and alcohol, 10 c.c., are mixed together with 5 c.c. of concentrated formalin to which distilled water is added up to 200 c.c., so as to make an orange-red coloured solution free from turbidity.

(*d*) Carbol fuchsin solution.

Procedure.

1. Thin films are made and allowed to dry in air for 1 to 7 days before staining.
2. Treat with acetone for 1 minute; wash slowly.
3. Mordant with formalin solution for 1½ to 2 minutes; wash with water.
4. Stain with carbol fuchsin solution for a few seconds; wash with water, dry, and examine.

Method two.

Solutions required.

(*a*) Sodium hydroxide, 0·5 gm., sodium carbonate, 0·5 gm., and distilled water, 10 c.c., are mixed together; absolute alcohol is added up to 100 c.c.

(*b*) Pure sulphuric acid, 2 c.c., water, 10 c.c., and absolute alcohol up to 100 c.c. are mixed together.

(*c*) Zinc or cadmium iodide, 5 gm., and iodine, 0·5. gm., are dissolved in absolute alcohol, 100 c.c.

(*d*) 1 per cent. aqueous solution of eosin.

(*e*) Ziehl-Neelsen's carbol fuchsin solution.

(*f*) Acetone.

Procedure.

1. Place in reagent (*a*) for 1 minute; wash in water.
2. Place in reagent (*b*) for 1 minute; do not wash in water.
3. Mordant in (*c*) for 1 minute; wash in water.
4. Treat with eosin solution (*d*) for 30 seconds; wash in water.
5. Stain in (*e*) for a few seconds; wash in water.
6. Differentiate in acetone for a few seconds; wash, dry, and examine.

The Paschen bodies of smallpox stain pink by this method.

Method three.

Solutions required.

(*a*) Sublimate-alcohol solution consisting of absolute alcohol, 30 c.c.; mercuric chloride, 4 gm.; and distilled water, 60 c.c.
(*b*) This consists of a solution of alcohol to which a trace of iodine has been added.
(*c*) 0·25 per cent. sodium thiosulphate solution.
(*d*) 2·5 per cent. iron alum solution.
(*e*) Heidenhain's iron haematoxylin solution.

Procedure.

1. Place in sublimate-alcohol at 60° C. for 3 hours; wash in water for 5 minutes.
2. Treat with iodine alcohol for 5 minutes; wash with water for 5 minutes.
3. Treat with sodium thiosulphate for 10 minutes; wash in water for 5 minutes.
4. Stain in Heidenhain's iron haematoxylin solution for 24 hours.
5. Differentiate in iron alum.

The elementary bodies appear greyish-blue in colour.

Method four.

Solutions required.

(*a*), (*b*), and (*c*) as in Method three.
(*d*) Either acid fuchsin solution is used consisting of 10 per cent. acid fuchsin containing 5 per cent. carbolic acid; or else 2 per cent. aqueous fuchsin containing 1 per cent. sulphuric acid is necessary.
(*e*) Alcoholic solution of picric acid.

Procedure.

1, 2, and 3 as in Method three.
4. Flood the slide with acid fuchsin and heat until the steam rises; allow to cool and repeat.
5. Differentiate in an alcoholic solution of picric acid.

The elementary bodies stain reddish-violet.

Method five (Gram's stain applied to elementary bodies).

Procedure.

1. Fix film in ether-alcohol for 20 minutes; wash with water.
2. Stain with acid fuchsin solution as employed in Method four.
3. Differentiate in an alcoholic solution of picric acid.

Elementary bodies appear brownish-red.

Method six.

Solutions required.

(*a*) Sodium hydroxide, 0·5 gm., and sodium carbonate, 0·5 gm., are added to 10 c.c. of distilled water; the total volume is made up to 100 c.c. by addition of absolute alcohol.
(*b*) Pure sulphuric acid, 2 c.c., is mixed with 10 c.c. of water; absolute alcohol is added up to 100 c.c.
(*c*) Zinc or cadmium iodide, 5 gm., and iodine, 0·5 gm., are dissolved in 100 c.c. of absolute alcohol.
(*d*) Carbol methyl violet solution (Gram).
(*e*) Lugol's iodine (Gram).

Procedure.

1. Thin films are made on clean slides and allowed to dry in air for 24 to 48 hours.
2. Treat with solution (*a*) for 1 minute; wash in water.
3. Treat with solution (*b*) for 1 minute; do not wash in water.
4. Treat with solution (*c*) for 1 minute; wash with water.
5. Stain film with carbol methyl violet for 4 seconds, heat gently until steam rises; wash with water.
6. Treat with Lugol's iodine for 30 seconds; wash in water.
7. Differentiate with alcohol or acetone; wash with water, dry, and examine.

The elementary bodies stain blue-violet.

Goodpasture's (1925) Method

This stain was originally introduced for Negri bodies in sections of rabies material. Subsequently, Goodpasture (1925) modified the stain by the introduction of potassium permanganate solution as a mordant, and employed it for staining the elementary bodies of molluscum contagiosum.

Solutions required.

Stain. 20 per cent. alcohol, 100 c.c.; phenol (concentrated and pure) solution, 1 c.c.; aniline oil, 1 c.c.; and basic fuchsin, 0·5 gm. The stain should be made up each time before use.
Mordant. 1 per cent. potassium permanganate solution in distilled water is used.
Counterstain. A saturated solution of methylene blue in alcohol, 30 c.c., and a 1 : 10,000 dilution of potassium hydrate in distilled water, 100 c.c.

Procedure.

1. Films should be fixed in Zenker's fluid as the method is non-applicable to tissues fixed in Helly's fluid.
2. Mordant for 1 minute with potassium permanganate solution; wash thoroughly in water.
3. Stain with carbol-aniline-fuchsin solution for 10 minutes.
4. Wash off the excess stain with water; blot with filter paper, and decolorize with 96 per cent. alcohol until the section or film turns light pink in colour.
5. Wash with water, and counterstain for 15 to 60 seconds with Löffler's methylene blue solution.
6. Wash with water, dehydrate, and decolorize for a few seconds in absolute alcohol until the excess of blue is removed.
7. Clear in xylol and mount in balsam.

Morosow's (1926) Modification of Fontana's Method for Staining Elementary Bodies

Solutions required.

Fixative. This consists of acetic acid, 1 c.c.; 40 per cent. formalin, 2 c.c.; and distilled water, 100 c.c.

Mordant. Carbolic acid saturated solution *Karbolsäure* (see Morosow, 1926); tannin, 5 gm.; distilled water, 100 c.c.

Silver stain. 20 c.c. of distilled water plus one drop of 25 per cent. ammonia are placed in a test-tube, and a 10 per cent. solution of silver nitrate is added, drop by drop with a pipette, until a brown precipitate appears. The addition of silver nitrate is continued until this precipitate redissolves and the solution turns opalescent.

Procedure.

1. Make a thin film.
2. Dry in air.
3. Place in distilled water for 10 to 15 minutes.
4. Dry in air.
5. Treat with fixative for 1 minute.
6. Wash with distilled water.
7. Treat with mordant and heat gently for 30 to 60 seconds until the steam rises, but do not allow it to boil.
8. Wash thoroughly with distilled water for 30 seconds.
9. Add about 0·5 c.c. of silver nitrate to the slide and again heat gently for 1 to 2 minutes until the film turns light brown in colour.
10. Wash thoroughly with distilled water and examine for Paschen bodies.

In a well-stained preparation elementary bodies should appear dark brown. In our experience this method has given good results, but we have come to the conclusion that caution is necessary as artefacts simulating virus bodies are liable to be found if the preparation has been overstained. Turevich (1935) has employed silver impregnation methods for staining the elementary bodies of vaccinia.

REFERENCES

Bedson, S. P. (1937), *Rep. publ. Hlth. med. Subj.*, Lond., no. 80.

Buist, J. B. (1887), *Vaccinia and Variola; a Study of their Life-history*, Churchill, London.

Castaneda, M. R. (1930), *J. infect. Dis.*, **47**, 416.

Coles, A. C. (1935), *J. R. micr. Soc.*, **55**, 249.

Craigie, J. (1933), *J. Path. Bact.*, **36**, 185.

Giemsa, G. (1910), *Münch. med. Wschr.*, **57**, 2476.

Goodpasture, E. W. (1925), *Amer. J. Path.*, **1**, 547.

Gordon, M. H. (1937), *Edinb. med. J.*, **44**, 65.

Gutstein, M. (1937), *J. Path. Bact.*, **45**, 313.

Haagen, E., and Kodama, M. (1934), *Zbl. Bakt.*, Abt. I, orig., **133**, 23.

Herzberg, K. (1934), *Zbl. Bakt.*, Abt. I, orig., **131**, 358.

—— (1936), *Klin. Wschr.*, **2**, 1385, 1665.

Mackie, T. J., and van Rooyen, C. E. (1937), *Edinb. med. J.*, **44**, 72.

Morosow, M. A. (1926), *Zbl. Bakt.*, Abt. I, orig., **100**, 385.

Paschen, E. (1906), *Münch. med. Wschr.*, **53**, 2391.

—— (1913), *Dtsch. med. Wschr.*, **39**, 2135.

Plimmer, H. G. (1913), *Proc. roy. Soc.*, B., **86**, 377.

Taniguchi, T., Hosokawa, M., Kuga, S., and Fujino, T. (1934 *a*), *Jap. J. exp. Med.*, **12**, 91.

Taniguchi, T., Hosokawa, M., Kuga, S., Komura, Y., and Nakamura, F. (1932), *Jap. J. exp. Med.*, **10**, 581.

Taniguchi, T., Hosokawa, M., Kuga, S., and Masuda, Z. (1934 *b*), *Jap J. exp. Med.*, **12**, 101.

Turevich, E. I. (1935), *Giorn. d. Batter. e Immunol.*, **14**, 1233.

van Rooyen, C. E. (1937), *Zbl. Bakt.*, Abt. I, orig., **139**, 130.

von Prowazek, S. (1905), *Arb. GesundhAmt.*, Berl., **22**, 535.

—— (1912), *Handbuch der pathogenen Protozoen*, Barth, Leipzig.

Zinsser, H., and Bayne-Jones, S. (1934), *A Textbook of Bacteriology*, p. 745, 7th ed., Appleton-Century, London.

Nicht einzeln im Buchhandel. *Ueberreicht vom Verfasser.*

Sonderabdruck aus dem

Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten.

I. Abteilung. Originale.

1937. Bd. 139.

Verlag von Gustav Fischer in Jena.

Printed in Germany.

[From the Bacteriology Department, Edinburgh University.]

Elementary (Paschen) Bodies in infectious Myxomatosis of the rabbit (Virus myxomatosum Sanarelli).

by **C. E. van Rooyen, M. D.**

With 5 figures in the text.

Various workers have described specific bodies and cellular changes associated with this condition. Splendore (1909) drew attention to the presence of "trachoma like" bodies in affected cells, but Moses (1911) was unable to confirm this observation. He showed that the infective agent was capable of passing certain filters able to withhold bacteria. Aragão (1911) described the existence of inclusion bodies within the hypertrophied nuclei of tumour cells, which he called Chlamydozoa myxomae. The same worker (1927) later withdrew this claim and suggested that the infective agent was a small round granule which appeared in the cytoplasm of infected cells, for which he proposed the name Strongyloplasma myxomae. Lipschütz (1927) likewise noticed similar bodies within the cytoplasm of swollen tumour

cells but disagreed that they were either strongyloplasms or chlamydozoa, and named them Sanarellia cuniculi. Rivers (1927) also reported acidophilic intracytoplasmic inclusion bodies in cells overlying the myxomatous masses and Hobbs (1928) confirmed these observations. Findlay (1929) on the other hand, failed to find inclusion material though he successfully confirmed Lipschütz's observations.

Granules similar to those described by Lipschütz have also been noted by Lewis and Gardner (1932) who demonstrated their presence in stellate and epithelioid cells by employing a modified Wright's stain. They, however, stated that these structures resembled the Rickettsia of heart water disease of sheep, and make no reference to virus particles. Later, Hyde and Gardner (1933) and Hyde (1936) suggested that the inclusion body of myxomatosis consists of a number of granules which probably represent the virus.

In this publication the writer desires to record the presence of minute bodies which he has observed extracellularly, in the conjunctival exudate of rabbits infected with myxoma virus. A specimen of rabbits testis infected with myxoma virus and preserved in 50 % glycerol solution was used for the investigations. A fragment of tissue 0.5 gram in weight was washed free of glycerol with physiological saline and ground in a mortar in 4.5 ccs of sterile broth. The emulsion was then plated out on blood agar medium and inoculated into a meat medium and incubated at 37° C for 10 days for evidence of cultivable aerobic or anaerobic bacteria. No growth was obtained, however, and the material was used for animal inoculation.

Experimental work.

Twelve healthy rabbits were selected and films of conjunctival secretion made from each eye prior to infection in order to serve as controls in each case. Six were inoculated intratesticularly and the remainder, by placing a drop of the material on the conjunctiva and then scarifying it with the point of a needle. All the animals died in 4 to 7 days. Prior to death, six of the rabbits which had been inoculated by the ocular route showed marked conjunctivitis accompanied by a profuse white muco-purulent exudate and three of the remainder showed similar eye lesions.

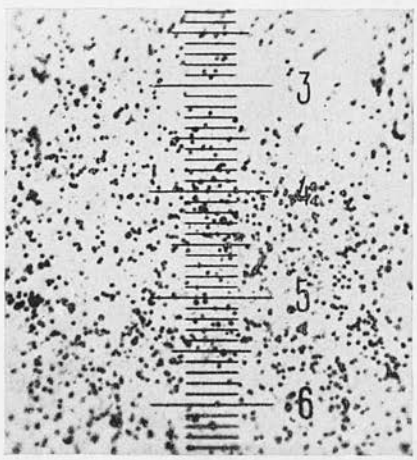


Fig. 1. Film made from the conjunctival secretion of a rabbit 4 days after inoculation with myxoma virus. Stained by Paschen's method. X 1222.

Direct films were made from conjunctival secretion obtained from each animal, on glass slides of 1.1 mm in thickness, and then stained by Paschen's method for the demonstration of elementary bodies. Briefly the technique consisted of the following procedure:

Air dried films were immersed in distilled water for 5 mins.; fixed in methyl alcohol for 3 mins., permitted to dry; treated with Löffler's flagella mordant for 5 mins., being gently heated for ½ min., thoroughly washed in distilled water for 1 min., and next stained with 5 % carbol fuchsin for 5 mins., being gently heated until steam arose. The stain was thereafter poured off, the film was rapidly washed in water, dried in hot air and mounted with a No. 1 coverslip in Hyrax (Watson) medium

possessing a refractive index of 1.8 when dried. The slide and coverslip were next placed between two heavy weights and the coverslip firmly compressed against the slide for a period of 24 hrs.

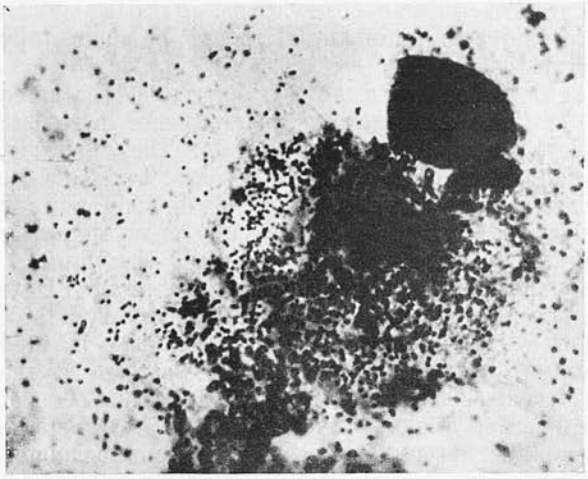


Fig. 2. Similar specimen to Fig. 1, showing a clump of elementary bodies and the remains of a ruptured cell. x 1222.

Preparations scrutinized under low power magnification failed to reveal any significant findings, but when examined under a x 1080 magnification several interesting observations were made.

The following optical system was employed for the purpose — x 13 Compensating ocular (Leitz), 2 mm apochromatic oil immersion objective of numerical aperture (N.A.) 1.3 (Zeiss), Holoscopic oil immersion substage condenser of N.A. 1.3 (Watson) which was used at full aperture throughout, and a 48 c.p. intensity source of illumination fitted with condenser, iris diaphragm and ground glass screen. Before each slide was examined the lamp filament, its diaphragm and the microscope substage condenser were accurately centred. Also, any necessary corrections were made to the length of the draw tube in order to compensate for slight variations in slide or coverslip thickness.

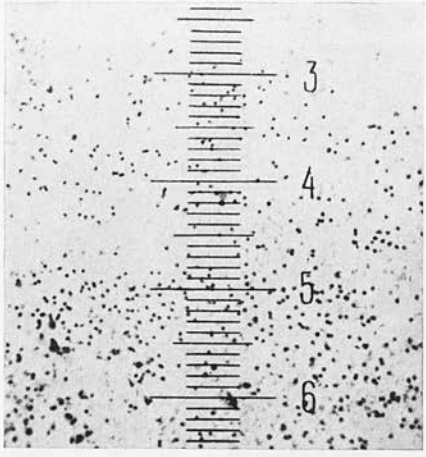


Fig. 3. Photomicrograph of a 3 days culture of vaccinia virus grown in the chicken embryo. x 1222.

With this optical equipment, it was possible to show the presence of numerous elementary bodies in every specimen of conjunctival exudate examined. These varied from 0.2 μ to 0.4 μ in diameter when measured by direct micrometry and from 0.31 μ to 0.36 μ when estimated by Nelson's (1909) method of measurement by extinction. The latter readings were obtained with a dry 6 L objective (Leitz) of maximum N.A. 0.65, fitted with an iris diaphragm and flange upon which graduations of N.A. were engraved. The instrument itself had been calibrated with an apertometer into eleven divisions of 0.05 ranging from N.A. 0.65 to N.A. 0.1 and each figure thus represented the specified N.A. Fig. 5, shows another objective designed for the same purpose. In order to reduce the possibility of personal error associated with this form of measurement, the results given represent the mean of two sets of readings obtained by the author and a colleague. Both white light and green were employed for the purpose (see Nelson 1909).

The number of virus bodies found in each specimen varied considerably but they appeared most numerous after the fourth day following inoculation of the virus by the conjunctival route. Morphologically, they frequently occurred in pairs and often appeared in chains of four or five (Fig. 1). Tetrad formation was never seen and variation in size seldom occurred. Often it was possible to demonstrate that the bodies were contained within epithelial cells and appeared in large groups in the proximity of cells which had been ruptured during fixation (Fig. 2).

These structures bear the closest possible resemblance to the elementary or Paschen bodies of vaccinia (Fig. 3) and there is little doubt that they are "virus bodies". Control films made from animals before infection failed to show such bodies.

An endeavour was next made to discover whether any significant differences could be distinguished between a specimen of Paschen bodies obtained from vaccinial keratitis of the rabbit and infectious myxomatosis affecting the same situation. After prolonged study of many specimens the conclusion has been drawn that: The elementary bodies of infectious myxomatosis are more numerous and stain more easily in conjunctival secretion than vaccinial elementary bodies. Short chain formation is also a feature and individual granules appear to be larger than Paschen bodies, which were 0.25 to 0.3 μ in size when stained and measured by the same methods.

It is difficult however to make positive statements regarding the size of such minute structures. Notwithstanding every possible precaution which may be taken to ensure that the particles are being viewed under identical conditions, there still exist a number of uncontrollable factors which are capable of influencing their apparent dimensions. For example, the estimated size is affected by the following factors: 1. Variations in the amount of stain deposited, especially when a mordant has been employed. 2. The presence of particles lying at different focal planes irrespective of how closely the coverslip and slide

Fig. 4. For purpose of comparison this illustration shows the appearance of staphylococci and B. coli when stained and photographed under identical conditions to Figs, 1, 2 and 3. x 1222.
(The above specimens have been stained by Paschen's method and are magnified x 1222. Each division on the micrometer scale in Figs 1, 3 and 4 = 0.14 μ. All the photomicrographs have been taken under precisely similar optical conditions and are therefore comparable with each other.)

Fig. 5. Photograph of an objective fitted with iris diaphragm and graduated flange, used for the measurement of minute objects by extinction.

have been brought into apposition. The apparent size of the elementary body can also be increased or diminished at will by varying the aperture of the iris diaphragm and N.A. of the condenser. Thus, the smaller the cone of light emerging from the condenser, the greater will be the amount of diffraction produced around the particle and, consequently, the larger will it appear to be. Although differences in estimated size due to this source of variation are extremely small in amount, they must nevertheless be borne in mind. To eliminate this fallacy a fixed (maximum) aperture of N.A. 1.3 has been employed throughout the present work. For purposes of comparison a third photograph (Fig. 4) taken under identical conditions to Nos. 1 and 2 and 3, showing B. coli and Staphylococci stained by Paschen's method, has been included.

Observations under dark-ground illumination.

Only stained and mounted preparations such as those described above have been examined by dark-field illumination. Unstained films have not been viewed by oblique light, because past experience indicated that conclusions based on such examination were more often misleading than helpful. The writer has therefore used dark-field illumination only to determine whether or not a particular elementary body, or group of particles noticed in a stained film were also refractile when seen by dark-ground. The means by which it has been possible to make such observations has been by applying the excellent principle advocated by Coles (1929). A slide is first examined with a 2 mm. oil immersion objective with a X 8 eyepiece and a typical staining elementary body moved into the centre of the field. The objective is then racked off the slide, the oil wiped off without moving the slide (which should be firmly fixed); an 8 mm apochromatic objective (Zeiss) is turned into position; a X 25 compensating ocular (Zeiss) substituted for the previous eyepiece; and a patch or Travis's expanding stop inserted into the substage condenser ring. The ground glass screen of the lamp is next removed and after refocussing upon the slide, dark-ground illumination should be immediately obtained. It should now be possible to know whether or not the stained granule previously seen, is also refractile to transmitted light. This proceeding should be repeated until several hundred elementary bodies have been viewed by this critical method of inspection.

From his own observations the writer is convinced that the elementary bodies reported in myxomatosis infection of the rabbit bear a close resemblance to the Paschen bodies of vaccinia.

A similar conclusion was reached by Aragão (1927) and the writer has therefore compared his own illustrations with those contained in Aragão's paper.

Although there is a close similarity between the elementary bodies described by the writer and Aragão's Strongyloplasma myxomae, it is impossible to assume their identity, for Aragão only makes a short reference to their presence and does not mention their size. Aragão furthermore used Giemsa's stain, whereas the writer used Paschen's stain for elementary bodies, and also obtained successful results with Victoria Blau 4 R stain (see Herzberg, 1934).

Control experiments.

Isolation of animals.

Since infectious myxomatosis of the rabbit is a highly contagious disease, all animals inoculated with the virus were housed in an isolation room in the laboratory. It should also be mentioned that there were no rabbits infected

with any other virus diseases present amongst the laboratory stocks, at the time when the experiments with myxomatosis virus were in course of progress.

Appearance of normal conjunctival secretion.

Films were made from the conjunctival secretion of 50 rabbits comprising 25 healthy normals and 25 experimental animals. The latter were being used in the laboratory for other investigations.

Each specimen was stained by Paschen's method for demonstration of elementary bodies and next examined by direct and dark-ground illumination as before. With each batch of slides a film of myxomatosis and vaccinia elementary bodies were included in order to control the efficiency of the staining technique.

In no single case was it possible to demonstrate in the control series elementary bodies similar to those found in myxomatosis and vaccinia infection. Occasionally in some films deeply stained granules of about 0.25 μ in size which resembled virus bodies were seen. Close inspection of the latter, often revealed that they were scanty in numbers, possessed a hazy indistinct outline and when viewed under dark-ground illumination failed to show refractility. In the same control films these could be demonstrated in another way. Namely, by first observing the specimen with oblique light and then by direct light, thus reversing the previous sequence of events.

By this method it was often observed that stained films of normal eye secretion when viewed by dark-field, appeared to be full of elementary bodies, judging by the multitude of refractile particles present. But when a field showing this appearance was viewed by direct light, stained elementary bodies could not be found at the spots at which the refractile granules were previously noted.

From these observations it was concluded that the non-staining refractile particles present in normal conjunctival secretion were probably artifacts.

The difference between the latter and true elementary bodies such as those of vaccina and myxomatosis, is even more striking when compared with each other, for elementary bodies not only stain readily by Paschen's method but are also refractile to oblique illumination.

The results therefore emphasize the caution necessary when interpreting the appearances seen by dark field illumination, which should invariably be made in conjunction with stained slides of the same specimen if possible. They moreover show the advantages to be gained by use of the optical system employed, which makes it possible to obtain dark or direct illumination of a stained specimen without moving the field.

Use of rabbits immunised against vaccinia virus.

Vaccinia-immune rabbits were employed in order to prove that the strain of myxomatosis virus studied was not contaminated with vaccinia virus.

Three rabbits which had been used for preparation of vaccinia antiserum were tested. These animals had received graded doses of virulent vaccinia virus for a period of 2 to 3 months and a fortnight before inoculation with myxomatosis virus they withstood 0.5 cc of 1.10 dilution of vaccinia virus in rabbits testis which was active to an end titre of 1 : 1000000 dilution. The three animals were next injected subcutaneously with 0.25 cc of a 1 : 1000000 dilution of myxomatosis virus contained in rabbit testicle tissue and then placed in separate cages. The first rabbit died in 5 days, the second in 9, and the third in 10 days. All the animals showed evidence of severe conjunctival infection accompanied by profuse secretion, in which countles myxomatosis elementary bodies were demonstrated.

Experiments on the guinea pig, rat, ferret, pigeon, hen, dog and pig.

Myxomatosis virus is non-pathogenic to the above species of animals (see Rivers 1930). Vaccinia virus on the other hand is infective in varying degree for the same animals. Two guinea pigs, two rats, two ferrets, one pigeon, one hen, one pig and a young dog, were inoculated subcutaneously, as well as by the ocular route, with 1 cc of a 1 : 10 dilution of myxoma virus active to an end titre of 1 : 1000000. The animals were kept under observation for two weeks but no evidence of disease was demonstrable at the end of this period. The susceptibility of these species to vaccinia virus was next tested by inoculating each of them with 0.25 cc of a 1 : 1000 dilution of vaccinia virus, and the material administered intraconjunctivally as well as by corneal scarification. Within seven days the two rats, two guinea pigs, dog and pig showed evidence of vesiculation at the site of inoculation accompanied by keratitis and general illness. The pigeon only showed transient cloudiness of the inoculated eye and the hen and ferrets failed to react.

The evidence thus indicates that the strain of myxoma virus employed in these experiments was free from contamination with vaccinia virus.

Summary.

The similarity of the elementary bodies found in myxoma infection to the Paschen bodies of vaccinia virus has made it essential to institute an elaborate series of experiments to establish the purity of the strain of myxomatosis virus used.

Control observations have proved that the elementary bodies found in myxomatosis are absent from the conjunctival secretion of animals before inoculation. They were also absent from the conjunctival secretion of 50 other rabbits chosen at random from normal stocks as well as experimental animals employed in the laboratory for other purposes. Two methods have been used to prove the absence of vaccinia virus from the strain of myxomatosis virus employed. First it was found impossible to infect rabbits immunised against vaccinia virus with 0.5 cc's a 1 : 10 dilution of this virus, but these rabbits were proved to be susceptible to 0.25 cc of a 1 : 1000000 dilution of myxoma virus. Secondly, the writer has demonstrated resistance in seven different species of animals to myxoma virus and has proved the susceptibility of the same animals to a stock laboratory strain of vaccinia virus. Much further work is necessary, however, before it can be finally suggested that the elementary bodies described in the present note are aetiologically related to myxomatosis of the rabbit. Serological and other studies with this object in view are in progress and will be reported later.

The aim of the present communication is to record in myxomatosis of the rabbit, the presence of typical elementary bodies resembling the Paschen bodies of vaccinia.

Conclusion.

1. Typical elementary bodies resembling in morphology the elementary or Paschen bodies of vaccinia have been demonstrated in infectious myxomatosis of the rabbit.

2. The size of these stained elementary bodies vary from 0.31 to 0.36 μ, when measured both by direct micrometry and by the extinction method. Vaccinial elementary bodies obtained from a similar source when stained and measured under identical conditions vary from 0.25 μ to 0.3 μ in diameter.

3. It is suggested that the myxomatosis elementary bodies are larger in size than vaccinial Paschen bodies; appear in greater numbers and occasionally show evidence of short chain formation.

4. Experiments on rabbits immunised against vaccinia virus as well as tests on animals of seven different species have proved that the strain of myxoma virus used for study was free from contamination with vaccinia virus.

Acknowledgements.

I desire to acknowledge my indebtedness to the late Professor Paschen as well as to Professor Nauck of Hamburg for the help and instruction they have given me in the staining and recognition of elementary bodies. I also wish to thank Dr. Gordon of St. Bartholomew's Hospital London and Professor Mackie of Edinburgh University for many helpful suggestions.

References.

Aragão, H. B., Brazil Med. **25**, 471 (1911). — Aragão, H. B., da Rocha Lima, H., a. v. Prowazek, Handb. d. pathog. Protoz., Leipzig **11**, 959 (1920). — Id., Mem. Inst. Cruz (port.) **20**, 237 (1927). — Coles, A. C., Brit. med. J. **11**, 91 (1929). — Findlay, G. M., Brit. J. exper. Path. **10**, 214 (1929). — Herzberg, K., Zbl. Bakter. I Orig. **131**, 358 (1934). — Hobbs, J. R., Amer. J. Hyg. **8**, 800 (1928). — Hyde, K. E., Ibid. **23**, 278 (1936). — Hyde, R. R., a. Gardner, R. E., Ibid. **17**, 446 (1933). — Lewis, M. R., a. Gardner, R. E., Amer. J. Path. **8**, 583 (1932). — Lipschütz, B., Wien. klin. Wschr. **1927**, Nr 40, 1101 — Moses, A., Mem. Inst. Cruz (port.) **3**, 46 (1911). — Nelson, E. M., J. Roy. Micro. Soc. 549 (1909). — Rivers, T. M., Proc. Soc. exper. Biol. a. Med. **24**, 435 (1927). — Id., J. of exper. Med. **51**, 965 (1930). — Splendore, A., Zbl. Bakter. I Orig. **48**, 300 (1909).

VOLPINO, G. (1909), *Zbl. Bakt.*, Abt. I, orig., **49**, 197.
—— (1912), *Rev. int. Vacc.*, **2**, 312.
VOSS, J. A. (1938), *Bull. Off. int. Hyg. publ.*, **30**, 1712.
WALTHARD, B. (1926), *Schweiz. med. Wschr.*, **56**, 854.
WANKLYN, W. McC. (1913), *How to diagnose Smallpox*, Smith Elder, London.
WARD, H. K. (1929), *J. exp. Med.*, **50**, 31.
WARD, H. K., and TANG, F. F. (1929), *J. exp. Med.*, **49**, 1.
WARLOMONT (1883), cited by Blaxall (1930).
VON WASIELEWSKI (1897), *Zbl. Bakt.*, **21**, 901.
—— (1901), *Z. Hyg. InfektKr.*, **38**, 212.
WATANABE, N. (1924), *Arch. Hyg., Berl.*, **92**, 359.
WEICHSEL, M. (1931), *Mschr. Kinderheilk.*, **49**, 28.
WEIGERT, C. (1874), *Anatomische Beiträge zur Lehre von den Pocken*, Breslau.
WESTPHAL, C. (1872), *Arch. Psychiat. Nervenkr.*, **3**, 376.
—— (1874), *Arch. Psychiat. Nervenkr.*, **4**, 338.
WHIPHAM, T., and MYERS, A. T. (1886), *Trans. clin. Soc. Lond.*, **19**, 164.
WIDELOCK, D. (1938), *J. infect. Dis.*, **62**, 27.
WILSON, R. E., and FORD, F. R. (1927), *Johns Hopk. Hosp. Bull.*, **40**, 337.
WINKLER, (1927), *Arch. Hyg. Berl.*, **98**, 241.
WINNICOTT, D. W., and GIBBS, N. (1926), *Brit. J. Child. Dis.*, **23**, 107.
WISHART, F. O., and CRAIGIE, J. (1936), *J. exp. Med.*, **64**, 831.
WOHLRAB, A. (1872), *Arch. Heilk.*, **13**, 512.
WOLF, H., and BRAMS, W. A. (1930), *J. nerv. ment. Dis.*, **71**, 714.
WOLLNITZA, E. (1938), *Arch. Derm. Syph., Wien*, **177**, 186.
WOODCOCK, H. M. (1921), *J. R. Army med. Cps.*, **37**, 418.
WOODRUFF, C. E. (1930), *Amer. J. Path.*, **6**, 169.
WOOODWARD, S. B. (1930), *New Engl. J. Med.*, **202**, 122.
—— (1932), *New Engl. J. Med.*, **206**, 570.
—— (1933), *New Engl. J. Med.*, **208**, 641.
WOODWARD, S. B., and FEEMSTER, R. F. (1933), *New Engl. J. Med.*, **208**, 317.
WOOLPERT, O. C. (1936), *Amer. J. Path.*, **12**, 141.
WOOLPERT, O. C., and GALLAGHER, F. W. (1937), *J. Bact.*, **33**, 58.
WRIGHT, O. K. (1935), *Bull. Hyg., Lond.*, **10**, 483.
WURTZ, R., TEISSER, P., CAMUS, L., TANON, L., and MARIE, P. (1913), *Bull. Acad. Méd., Paris*, **69**, 65.
XYLANDER (1909), *Zbl. Bakt.*, Abt. I, orig., **51**, 290.
YAOI, H. (1931), *Bull. Off. int. Hyg. publ.*, **23**, 1807.
—— (1934), *Jap. J. exp. Med.*, **12**, 123.
—— (1935 *a*), *Jap. J. exp. Med.*, **13**, 307.
—— (1935 *b*), *Bull. Off. int. Hyg. publ.*, **27**, 277.
—— (1936 *a*), *Bull. Off. int. Hyg. publ.*, **28**, 1933.
—— (1936 *b*), *Bull. Off. int. Hyg. publ.*, **28**, 1939.
—— (1936 *c*), *Jap. J. exp. Med.*, **14**, 311.
—— (1937), *Jap. J. exp. Med.*, **15**, 155.
—— (1938 *a*), *Jap. J. exp. Med.*, **16**, 117.
—— (1938 *b*), *Paris méd.*, **28** (2), 127.
YAOI, H., and AKASAWA, S. (1938), *Jap. J. exp. Med.*, **16**, 465.
YAOI, H., and KASAI, H. (1929 *a*), *Jap. J. exp. Med.*, **7**, 241.
—— —— (1929 *b*), *Jap. J. exp. Med.*, **7**, 243.
—— —— (1929 *c*), *Jap. J. exp. Med.*, **7**, 389.
—— —— (1929 *d*), *Jap. J. exp. Med.*, **7**, 579.
—— —— (1929 *e*), *Jap. J. exp. Med.*, **7**, 585.
—— —— (1930), *Jap. J. exp. Med.*, **8**, 499.
—— —— (1931 *a*), *Bull. Off. int. Hyg. publ.*, **23**, 299.
—— —— (1931 *b*), *Jap. J. exp. Med.*, **9**, 619.
—— —— (1933), *Jap. J. exp. Med.*, **11**, 383.
YAOI, H., and NAKAHARA, W. (1934), *Jap. J. exp. Med.*, **12**, 131.
YOANNOVITCH (1930), *Bull. Off. int. Hyg. publ.*, **22**, 1153.
YOFFEY, J. M., and SULLIVAN, E. R. (1939), *J. exp. Med.*, **69**, 133.
ZELLER, H., GILDEMEISTER, E., and HILGERS, P. (1933), *Zbl. Bakt.*, Abt. I, orig., **128**, 21.
ZUELZER, W. (1874), *Zbl. med. Wiss.*, **12**, 82.
ZURUKZOGLU, ST. (1927), *Klin. Wschr.*, **6**, 70.

CHAPTER XXXI

RIFT VALLEY FEVER

Rift Valley fever or enzootic hepatitis was first described by Daubney, Hudson, and Garnham in 1931. These workers found the disease in Kenya Colony among sheep, and at the same time recorded its experimental transmission to man. In all probability the condition was noticed earlier by Montgomery (1913), who recorded an outbreak of a similar disease causing a 90 per cent. mortality among lambs at a government farm at Naivasha in Kenya. The 1930 outbreak, which was described by Daubney and his co-workers, occurred on a farm situated at an altitude of 5,500 to 6,000 feet extending northwards from the shores of Lake Naivasha in the Rift Valley. About 3,500 lambs and 1,200 ewes died from the disease, and the mortality attained a figure of 95 per cent. among the 3 to 7 days' old lambs (see also Findlay and Daubney, 1931).

Rift Valley fever has been noticed in Kenya, Uganda, Anglo-Egyptian Sudan, French Sudan, and French Equatorial Africa, but has not yet been reported in Nigeria, the Gold Coast, Sierra Leone, or Gambia (Findlay, Stefanopoulo, and MacCallum, 1936).

THE DISEASE IN MAN

Daubney, Hudson, and Garnham (1931) demonstrated the infectivity of Rift Valley fever virus for man by inoculating a human subject with a filtrate derived from infected sheep tissue. Later, Findlay (1932) recorded that many of the natives employed in herding sheep during the period of the enzootic also suffered from a short febrile condition resembling the experimental disease in man. Then, Schwentker and Rivers (1934) recorded a fatal case in a laboratory worker who contracted the disease and died following complication with thrombophlebitis. Kitchen (1934) described another laboratory infection, and subsequently Francis and Magill (1935) and Findlay (1932) drew attention to 6 further cases that were acquired in a similar fashion. Curasson (1934) has suggested the possible occurrence of the disease in the French Sudan. The general course of the malady is that of a mild febrile illness and, according to Daubney, Hudson, and Garnham, at least 200 human cases have occurred in British East Africa, without complications or death.

Clinical Features

After an incubation period of 5 to 6 days, the symptoms of the disease develop suddenly. A wide variety of clinical manifestations are liable to be present, such as general malaise, headache, nausea, vomiting, shivering, rigors, shooting pains in the muscles and joints, particularly affecting the shoulder region and back, a sense of fullness over the liver, tenderness of the eyeballs, photophobia, mental confusion, vertigo, and epistaxis. The pyrexia varies from about 101 to 103° F., is of the so-called 'saddle-back' type resembling that recorded in dengue, and may occasionally reach 105–6° F., the pulse rate being also increased. Usually, the disease only lasts for a few days and the convalescent period is short. Relapses have been reported (see Francis and Magill, 1935).

Clinical Pathology

The most characteristic feature is the presence of a leucopenia. A figure of 3,720 w.b.c. per c.mm. has been recorded by Francis and Magill, and

the decline principally affects the polymorphonuclear leucocytes. Findlay (1932) has recorded the presence of occasional myelocytes and polymorphonuclear leucocytes possessing vacuolated nuclei. He also states that the leucopenia may persist into convalescence. Throughout the disease the patient's urine may appear to be normal in its colour and constituents, containing neither blood, bile, nor casts. Findlay (1932), however, has mentioned a case in which the urine was of a deep yellow colour and was not pathogenic for mice. On the other hand, blood, or throat washings derived from patients during the height of the illness have proved infective for mice.

Protective antibodies have been detected in the blood serum of individuals 9 days after the onset of fever, and complement-fixing antibodies have likewise been shown in such sera by Broom and Findlay (1932, see also p. 89). Schwentker and Rivers (1934) found that, in the case of Rift Valley fever observed by them, the patient's serum neutralized over 1,000 m.l.d. of virus for mice.

Pathology

The only fatal case on record is that reported in a laboratory worker who died from venous thrombosis, 45 days after the onset of infection. The acute stage of his illness only occupied the first week. At autopsy, clots were present principally in the inferior vena cava, and to a lesser extent in the saphenous and femoral veins. Chronic pleuritis together with old pulmonary infarcts and emboli were present in both lungs. The liver showed no abnormalities, and the virus could not be recovered from post-mortem tissue. This was doubtless due to the active stage of the disease having subsided some considerable time prior to death.

THE NATURAL DISEASE IN SHEEP

This is of a variable character, and the following signs have been recorded: vomiting, diarrhoea, melaena, mucopurulent nasal discharge, loss of appetite, abortion in pregnant animals, unsteady gait, and death usually within 24 hours after the onset of symptoms. The temperature is often normal.

At post mortem the following changes are seen:

The liver. Focal necrosis of this organ is the most striking feature of the disease. Small white foci of necrosis about 1 mm. in diameter appear beneath the capsule of the liver, frequently surrounded by pin-point haemorrhages. Necrotic foci are evenly distributed throughout the organ, and when numerous they tend to coalesce, forming a diffuse necrotic lesion. There is, however, no sign of demarcation of necrotic areas from surrounding liver tissue. The liver is usually not increased in size but is occasionally engorged with blood.

The spleen is not enlarged, but shows numerous subcapsular pin-head haemorrhages, especially at the free border of the organ.

The kidneys. Haemorrhages and capillary arborescence have sometimes been recorded.

The lungs. Small, slightly raised areas which are oval in shape and an inch or more in diameter occur on the surface of the organ.

Circulatory system. Subpericardial ecchymoses are common in the region of the coronary grooves or the ventricles of the heart. The mesenteric vessels are always strongly injected and engorged with blood.

Lymphatic glands of the latter region may be enlarged and show cortical necrosis.

Alimentary tract. The lesions here are of variable character, the principal changes affecting the lower intestine being hyperaemia, congestion, and in severer cases, haemorrhagic enteritis.

CHARACTERISTICS OF THE VIRUS

Pathogenicity to Animals

Other than its infectivity to the lamb, sheep, and the human subject, the virus has been shown to be pathogenic to a number of other species of animals (see Findlay, 1932). In rhesus monkeys it produces a non-fatal illness, followed by recovery and protection from further attacks for at least 6 months afterwards.

The African monkeys, *Cercopithecus callitrichus*, *Erythrocebus patas*, and *Cercocebus fuliginosus*, fail to exhibit a febrile reaction when inoculated with Rift Valley virus, although it may persist for a few days in their blood. Findlay (1932 *a*) pointed out that the relative insusceptibility of these species to infection was not due to the presence of antibodies in their blood, since antibodies only developed after inoculation. South American monkeys, such as *Cebus fatuellus*, *C. chrysopus*, *Hapale jacchus*, and *H. penicillata*, were more susceptible and showed a febrile reaction following infection. The virus can also be recovered from the blood of these species during a pyrexial period, and immune bodies are demonstrable in the blood of capuchin monkeys 14 days after injection. Cats exhibit a transient febrile illness. The virus is highly pathogenic and proves invariably lethal to mice, field voles (*Microtus agrestis*), wood mice (*Apodemus sylvaticus*), dormice (*Muscardinus avellanarius*), and golden hamsters (*Cricetus* (*mesocricetus*) *aurantus*). In rats the virus causes a 50 per cent. mortality and is of about the same degree of pathogenicity to the ferret (see Francis and Magill, 1935). The cow, goat, and grey squirrel suffer from a severe but non-fatal malady. Rabbits appear to be resistant, but may harbour the virus for a short time in their blood after infection. The guinea-pig, mongoose, hedgehog, tortoise, frog, hen, pigeon, and parakeet are resistant. Susceptible animals can be infected by any route of inoculation, and in pregnant animals the virus has been shown to be capable of passing through the placenta into the foetal circulation.

Morbid Histology of the Infected Liver

Lesions present in the sheep, goat, rat, mouse, monkey, squirrel, hamster, vole, and wood mouse have been carefully studied by Findlay (1933). The changes are either those affecting the cytoplasm or the nuclei of liver cells, and are as follows:

Changes in the cytoplasm.

The initial lesion is focal necrosis of the liver, which may affect any area, but in the rhesus monkey it tends to be confined to the mid-zonal area of the lobule. In lambs, mice, rats, and other small rodents no such distinction is possible, owing to the rapidity with which the infection spreads through the whole organ. For example, in mice, 48 hours after inoculation, there is little or no recognizable liver tissue left. With rhesus monkeys the changes are less extensive and localized hyaline necrosis occurs, characterized by the formation of round, hyaline, acidophilic bodies within the cytoplasm of individual liver cells. These structures are often surrounded by a clear space and may even be extruded from the liver cell; they tend to be best developed in the most resistant animals such as the adult sheep and goat, whereas they are less well marked in the susceptible mouse.

Fatty degeneration and pigmentation are only occasionally seen in the rhesus monkey and are absent in other species. In the case of mice, 48 hours after infection the whole structure of the liver lobule is destroyed, nuclei show karyorrhexis, the cytoplasm becomes vacuolated and contains a few remnants of mitochondria and nuclear chromatin. The changes occurring in the Golgi apparatus were also investigated by Findlay, who employed the Mann-Kopsch method for the purpose, and found that all traces of Golgi apparatus had disappeared from the liver cells at the end of 48 hours in infected mice.

Nuclear changes.

These changes in liver cells are a characteristic feature of the disease. They were originally observed by Daubney and his co-workers, and later extensively reinvestigated by Findlay (1932). The essential changes are margination of the chromatin which becomes distributed inside the periphery of the nuclear membrane, and the appearence of acidophilic material within the nucleoplasm. In fresh tissue the latter may be stained by vital dyes such as dilute water-soluble eosin, methylene blue, neutral red, or phloxine red. Janus green B and osmic acid exert no effect upon them. Findlay states that the intranuclear inclusions of Rift Valley fever are dissolved by 0·1 per cent. acetic acid or 1 per cent. ammonia ; moreover, he adds that their existence in fresh tissue militates against their being artefacts in origin. Chemical tests have failed to disclose the nature of their composition, as they are neither blackened by osmic acid, nor can they be stained with Sudan III, Scharlach R, or Nile Blue sulphate in frozen sections. They give a negative reaction for chromatin by the Feulgen technique, and also a negative Bensley MacCallum for masked iron. The following is a list of stains used by Findlay for demonstrating intranuclear inclusions of Rift Valley fever: iron haematoxylin and eosin, Giemsa, eosin and methylene blue, phloxine and methylene blue, resorcin fuchsin, phosphotungstic acid haemotoxylin, Mallory's stain, Castaneda's stain, neutral red and neutral gentian violet.

For work bearing on micro-incineration studies carried out in Rift Valley fever the reader is referred to the studies of Horning (1934).

Neurotropic Strain of Rift Valley Fever Virus

Mackenzie and Findlay (1936) passaged the virus 30 times intracerebrally through mice. By this method they succeeded in producing a neurotropic strain of virus which possessed a fixed affinity for nervous tissue, and such a strain, when injected intracerebrally in mice, produced encephalomyelitis without liver necrosis. Rhesus monkeys were also infected with this strain by intracerebral or intranasal inoculation. Rabbits and guinea-pigs were apparently insusceptible (Mackenzie, Findlay, and Stern, 1936).

Morphology, Size, and Filterability of the Virus

This is one of the smallest viruses known, and some of the earliest experiments indicated this fact. Thus Daubney and his colleagues found that it passed through the Pasteur-Chamberland L_{11} bougie, and occasionally even the L_{13} size. It also passed the Berkfeld V, N, and W grades of candle at a negative pressure of 630 mm. Hg. (Findlay, 1932). Subsequently its size was more accurately measured by Broom and Findlay (1933), who filtered it through Elford's gradocol membranes and estimated its dimensions to lie between 23 and 35 mμ. According to these figures, therefore, the virus is well beyond the limits of visibility of the ordinary

microscope, and is even too small to be photographed by the ultra-violet light instrument (Findlay, 1933). Likewise all attempts to demonstrate the virus by staining methods have been without success (Mackenzie, 1933).

Cultivation

Cultivation was first performed by Mackenzie (1933), who used the method of Li and Rivers (1930), and employed a culture medium consisting of minced chicken embryo fragments suspended in Tyrode's solution (see p. 108). Cultures were put up in 5 c.c. Erlenmeyer flasks containing 5 c.c. of extract, incubated at 37° C. for 3 days and subcultivated by transferring 0·2 c.c. of the growth to a fresh medium. In this way Mackenzie cultivated the virus through 13 passages, without either diminishing the infectivity of the material or altering its characteristics. Saddington (1934) has also succeeded in cultivating the virus *in vitro* in tissue cultures.

Preservation of Virus

Kitchen (1934) has preserved it for at least 8 months by mixing blood from ill mice with normal serum diluted 1/10, and then desiccating the mixture *in vacuo*. During the course of our own studies on this virus we have preserved it by desiccating mouse liver tissue *in vacuo* over calcium chloride, according to Craigie's method (see p. 87). Findlay (1932) showed that lamb's blood, to which 0·5 per cent. carbolic acid was added, stored at 4° C., retained its infectivity for mice for 6 months. After 8 months' storage in oxalate-carbolic-glycerol there was some loss of virulence.

Resistance to Heat

The thermal death point of the virus contained in blood has been ascertained to be 56° C. for 40 minutes (Findlay, 1932).

IMMUNITY REACTIONS IN MAN AND ANIMALS

It has already been mentioned that the sera of humans or animals which have recovered from an attack of Rift Valley fever show both the presence of virus-neutralizing as well as complement-fixing antibodies (see Broom and Findlay, 1932; also p. 89). The latter appear about 14 days after the date of infection, and the former have been shown to persist in human serum from 4 to 5 years afterwards (Findlay, 1936). This worker has also drawn attention to the fact that certain mixtures of Rift Valley fever virus and immune serum, which were apparently non-infective when injected intraperitoneally in mice, could be reactivated or dissociated by simple dilution with saline. The phenomenon was a quantitive one, and if falling dilutions of serum were added to equal amounts of undiluted immune serum, a point was reached at which the mixture could no longer be reactivated or dissociated by simple dilution. Findlay also showed that mixtures of virus and immune serum which were neutral on intraperitoneal injection proved to be pathogenic if administered intranasally. Likewise a non-infective mixture of immune serum and virus could be rendered infective by increasing its amount. The immunization of mice against Rift Valley fever virus has been described by Mackenzie (1935).

An interference phenomenon.

Findlay and MacCallum (1937) have described the existence of a most interesting phenomenon relating to the apparently antagonistic action of Rift Valley fever and yellow fever viruses when introduced simultaneously into the same animal. They have shown that the inoculation of a mixture

of both viruses into rhesus monkeys resulted in protection of 7 out of 10 animals. Likewise, a single inoculation of mice with neurotropic yellow fever virus and pantropic Rift Valley fever virus delayed their death and even saved a few from it. The protective action was absent, however, if Rift Valley fever virus was injected 24 hours previously. The effect of substituting fowl-pest virus (which is non-pathogenic to mice) was also tried in place of yellow fever virus, but this failed to afford the same degree of protection against Rift Valley fever infection.

In explanation of these findings, Findlay and MacCallum have suggested that, when certain cells are occupied by an actively multiplying virus, they cannot be invaded by other virus particles. They also draw an analogy between this phenomenon and the occurrence of a somewhat similar phenomenon in respect of plant viruses. In the case of plants, it has been shown that a feebly pathogenic agent will afford protection to a plant when infected with a more virulent one, provided that the two viruses are generically related; see Thung (1931), Salaman (1933), Kunkel (1934, 1936), Holmes (1934), Ainsworth (1934), Caldwell (1935), McKinney (1935), and Price (1935, 1936).

LABORATORY DIAGNOSIS OF RIFT VALLEY FEVER

During a febrile attack the patient's blood should be withdrawn and inoculated subcutaneously or intraperitoneally in a mouse. If the animal dies, its liver and blood should first be examined to exclude the presence of bacteria, by aerobic and anaerobic methods of cultivation. If the result be negative, then histological sections of liver should be stained and searched for presence of intranuclear inclusion bodies and other changes characteristic of Rift Valley fever. Should the patient's blood prove non-infective to mice, then the individual's serum should be tested for the existence of virus-neutralizing antibodies.

RELATIONSHIP TO OTHER VIRUS DISEASES

Rift Valley fever in man is of interest on account of its clinical similarities to dengue fever on the one hand and epidemic influenza on the other. Pathologically, moreover, the characteristic liver necrosis evident in Rift Valley infection of laboratory animals bears a striking resemblance to human liver necrosis caused by yellow fever virus. There is, however, no reason to believe that Rift Valley fever produces extensive liver lesions in man; at least, in those cases which have been quoted in the literature, it has not caused sufficient damage in order to be recognizable clinically.

Dengue fever.

With regard to dengue fever and its possible relationship to Rift Valley fever, Findlay (1932 *b*) investigated this problem and found that both conditions were entirely different and separate pathological entities. Thus he successfully demonstrated, by means of cross-neutralization tests, that the monkey *Silenus rhesus* when immunized against dengue was not immune to Rift Valley virus. In the same way, monkeys immunized against Rift Valley fever could be infected with dengue virus. Also, mice injected with dengue virus were not immune to Rift Valley fever virus.

Yellow fever.

The points of similarity and dissimilarity existing between this and Rift Valley fever virus are summarized in an excellent account of the subject in the work of Findlay and MacCallum (1937). The features of

differentiation may be summarized thus: Both viruses are exceedingly small in size, but yellow fever virus, which measures 18 to 27 mμ (see Broom and Findlay, 1933; and Bauer and Hughes, 1935), is the smaller of the two; however, Findlay and MacCallum (1937) have accepted these figures with reserve, in view of the margin of error involved when estimating the size of the smaller viruses by ultra-filtration. Rift Valley fever virus is highly infective to sheep, goats, and small rodents, whereasy ellow fever virus does not attack these species. *In vitro* virus-neutralization tests performed with immune sera derived from either condition have failed to reveal the existence of cross-immunity between these agents. *In vivo* tests have shown that monkeys recovering from Rift Valley fever are susceptible to yellow fever virus and vice versa: moreover, in the case of a laboratory assistant, it was shown that the individual first contracted an attack of yellow fever and 3 years later developed an attack of Rift Valley fever. These viruses have many features in common, for example, clinically a case of Rift Valley fever in man resembles that of a mild attack of yellow fever.

Certain species of monkey appear to exhibit the same degree of susceptibility to both infections, according to Findlay (1932, 1933). In liver tissue, acidophilic intranuclear inclusions as well as Councilman lesions appear in both diseases. Likewise, virus possessing neurotropic properties is obtainable from either agent by repeated intracerebral passage (see Mackenzie, Findlay, and Stern, 1936).

Both yellow fever and Rift Valley fever confer lasting immunity to man following one attack. Like yellow fever, Rift Valley fever appears to be transmitted by mosquitoes of the aëdes genus (Daubney and Hudson, 1933), and Daubney and Hudson (1932) also showed that the virus was able to survive for 7 days in the engorged nymph of the species *Rhipicephalus appendiculatus*. In view of these findings, Findlay and MacCallum (1937) have ventured to suggest that it is conceivable that both these viruses may have originated from some common ancestral form, although geographically their distributions vary (see pp. 447 and 412). The problem of the classification of these two diseases has also been discussed by Broquet (1932).

Influenza.

The difficulty of differentiating Rift Valley fever from acute influenza on purely clinical grounds has been emphasized in a report by Francis and Magill (1935). Both diseases exhibit many features in common, for example, the abrupt onset, short incubation period, the presence of aches and muscular pains, together with a marked leucopenia in the peripheral blood.

In three cases of laboratory infection studied by Francis and Magill, the virus was recovered from the nasopharyngeal secretion of patients and transmitted to ferrets by intranasal instillation. The virus proved to be highly pathogenic to the ferret and caused not only pulmonary consolidation, but also liver necrosis and haemorrhages into other organs. The laboratory diagnosis was, however, confirmed by inoculating mice subcutaneously and examining histological sections of liver for the presence of intranuclear inclusions, Councilman lesions, necrosis and other changes.

Author's Observations on the Virus of Rift Valley Fever.

For a period of eighteen months I carried out systematic examinations on the tissues of mice infected with Rift Valley fever virus, for evidence of the presence of elementary bodies. The work was interrupted by the war, but the preliminary experiments were completed and evidence was obtained to show that the virus of Rift Valley fever was a typical elementary body which could be stained, demonstrated and photographed under the ordinary microscope. In size it measured about 70 mμ to 80 mμ according to micrometric extinction and by direct mensuration when compared with the elementary bodies of vaccinia against a micrometer scale, it was found to be appreciably smaller than the latter. They were usually found within cells of the reticulo-endothelial system and could be demonstrated in tissue scrapings prepared from the feet and blood vessels of infected mice. When examined by dark-field illumination they were highly refractive and were always present in the sera of infected animals and absent from uninoculated controls. These elementary bodies did not form characteristic clumps of inclusion bodies, and when they were observed inside cells they seemed to lie free and unsurrounded. Neither was there any data to suggest (as will be explained later) that the intranuclear inclusion bodies of Rift Valley fever were composed of the/

the elementary bodies I have described.

This observation (which I believe to be correct) illustrates how critical microscopic and staining methods could increase the size of one of the smallest of elementary bodies so as to make it visible under the microscope.

Microdissection of the Intranuclear Inclusion Bodies of Rift Valley Fever.

It is not yet known if the intranuclear inclusions of Rift Valley fever constitute virus aggregates or tissue derivatives of damaged cells.

To gain some information on this point I was able to make suitable preparations of intra-vitam stained infected liver tissue, and to dissect these cells under a micromanipulator.

My results showed quite clearly that the intranuclear inclusions of infected liver cells were very different from other virus inclusions which I had studied by the same technique, e.g., those of fowl-pox, ectromelia and molluscum contagiosum. As mentioned earlier, the former were not surrounded by definite membrane and on incising the outer covering of the liver cell nucleus the contained inclusion body disintegrated. Attempts to extract a single inclusion body and to inoculate a mouse with it were not possible, consequently/

consequently, with the aid of a micro-pipette I picked up a single infected liver cell nucleus, washed it in saline; and inoculated a mouse. This was repeated three times with negative results; although on each occasion the saline solution used for washing the inclusion was infective to a mouse. My results, therefore, indicate that the intranuclear inclusions in infected liver tissue in Rift Valley fever are not virus colonies but tissue derivatives instead. However, it would be imprudent to draw definite conclusions until further investigations can be performed, but the initial results appear to be significant. It is worth remarking that micromanipulation and dissection was undertaken at a magnification of 700 to a maximum of 1600 diameters, which is the highest limit of magnification at which such operations are possible.

REFERENCES

AINSWORTH, G. C. (1934), *Ann. Biol. appl.*, **21**, 581.
BAUER, J. H., and HUGHES, T. P. (1935), *Amer. J. Hyg.*, **21**, 101.
BROOM, J. C., and FINDLAY, G. M. (1932), *Lancet*, **1**, 609.
—— —— (1933), *Brit. J. exp. Path.*, **14**, 179.
BROQUET, C. (1932), *Pr. méd.*, **40**, 260.
CALDWELL, J. (1935), *Proc. roy. Soc.*, B, **117**, 120.
CURASSON, G. (1934), *Bull. Soc. Path. exot.*, **27**, 599.
DAUBNEY, R., HUDSON, J. R., and GARNHAM, P. C. (1931), *J. Path. Bact.* **34**, 545.
DAUBNEY, R., and HUDSON, J. R. (1932), *Lancet*, **1**, 611.
—— —— (1933), *E. Afr. med. J.*, **10**, 2.
FINDLAY, G. M. (1932), *Trans. R. Soc. trop. Med. Hyg.*, **25**, 229.
—— (1932 *a*), *Trans. R. Soc. trop. Med. Hyg.*, **26**, 161.
—— (1932 *b*), *Trans. R. Soc. trop. Med. Hyg.*, **26**, 157.
—— (1933), *Brit. J. exp. Path.*, **14**, 207.
—— (1936), *Brit. J. exp. Path.*, **17**, 89
FINDLAY, G. M., and DAUBNEY, R. (1931), *Lancet*, **2**, 1350.
FINDLAY, G. M., and MACCALLUM, F. O. (1937), *J. Path. Bact.*, **44**, 405.
FINDLAY, G. M., STEFANOPOULO, G. M., and MACCALLUM, F. O. (1936), *Bull. Soc. Path. exot.*, **29**, 986.
FRANCIS, T., and MAGILL, T. P. (1935), *J. exp. Med.*, **62**, 433.
HOLMES, F. O. (1934), *Phytopathology*, **24**, 845.
HORNING, E. S. (1934), *J.R. micr. Soc.*, **54**, 9.
KITCHEN, S. F. (1934), *Amer. J. trop. Med.*, **14**, 547.
KUNKEL, L. O. (1934), *Phytopathology*, **24**, 437.
LI, C. P., and RIVERS, T. M. (1930), *J. exp. Med.*, **52**, 465.
MACKENZIE, R. D. (1933), *J. Path. Bact.*, **37**, 75.
—— (1935), *J. Path. Bact.*, **40**, 65.
MACKENZIE, R. D., and FINDLAY, G. M. (1936), *Lancet*, **1**, 140.
MACKENZIE, R. D., FINDLAY, G. M., and STERN, R. O. (1936), *Brit. J. exp. Path.*, **17**, 352.
MCKINNEY, H. H. (1935), *Science*, **82**, 463.
MONTGOMERY, R. E. (1913), *Annu. Rep. Dep. Agric. Kenya*, 1912–13.
PRICE, W. C. (1935), *Phytopathology*, **25**, 776.
SADDINGTON, R. S. (1934), *Proc. Soc. exp. Biol., N.Y.*, **31**, 693.
SALAMAN, R. N. (1933), *Nature*, **131**, 468.
SCHWENTKER, F. F., and RIVERS, T. M. (1934), *J. exp. Med.*, **59**, 305.
THUNG, T. H. (1931), *Hand. ned.-ind. natuurw. Congr.*, **6**, 22–26 Sept., 450.

P.T.O →

CHAPTER XXXII

DENGUE FEVER

Historical

According to Hirsch (1883) this disease has received many different names. Thus the Spaniards called it *dengue*, corresponding in meaning to the French word *minauderie* and the English word *dandy*. The word *colorado* was also applied by the Spaniards as a descriptive term. The English and Americans have called it *break-bone* and *broken-wing*, the French *giraffe* and *bouquet* (whence the English corrupt *bucket*), and the Brazilians have named it *polka fever*.

Hirsch states that the earliest accounts of dengue fever were those given by Galberti of Egypt in 1779; by Bylon of Batavia in 1779; and by Rush of Philadelphia in 1780. Later, the disease appeared in the West Indies and shores of the Gulf of Mexico, where it was called dengue fever by the local physicians. It was not, however, until dengue broke out in the Eastern Hemisphere in 1871–3 that the disease received universal recognition as a distinct clinical entity.

EPIDEMIOLOGY

A list of the large epidemics occurring in various parts of the world is contained in a publication on the subject by the American authority Armstrong (1923), and these are stated to be as follows: Spain, 1793; Peru, 1818; India and Suez, 1825; U.S.A., Mexico, West Indies, and South America, 1826–8; India (Googeret), 1824–8; Arabia, 1835; India, 1836 and 1844; Bermuda, 1837; Egypt, 1845; India, 1847; Senegambia, 1845–8; Brazil, 1845–9; U.S.A. and West Indies, 1850–4; India, 1853–4; Tropics of Eastern Hemisphere, 1870–5; Louisiana (U.S.A.), 1872; Tripoli, 1878; Caribbean, North America, and Egypt, 1880; Caledonia, 1884–5; Fiji Islands, 1885; Texas (U.S.A.), 1885; Tripoli, 1887; Asia Minor, 1889–90; Texas, 1894 and 1897; Hawaii, 1903; Texas, 1907 and 1918; Bermuda, 1915; Egypt, 1916; Southern United States, 1922; New South Wales, 1925; Greece, 1927; Darwin, North Australia, 1927–8; and more recently in Miami, in 1934.

The rapidity with which dengue fever spreads is popularly regarded as being only second to that of epidemic influenza. For example, Armstrong (1923) states that at Austin, Texas (U.S.A.), in the epidemic of 1885, out of a population of 22,000 persons, 16,000 were attacked by the disease. At Cairo (in Egypt) in 1880 four-fifths of the inhabitants were affected, and during the 1818 epidemic at Lima in Peru only a few of its 70,000 inhabitants are said to have escaped infection, according to Hirsch (1883). In his article Armstrong (1923) records an outbreak of dengue observed by himself at Monroe, La. (U.S.A.), in 1922, among the poorer members of the negro community, during which 1,000 inhabitants were attacked even before the local health authorities became aware of its existence. This epidemic was terminated by a change in the climatic conditions and the advent of frost (see p. 434).

Within the last decade Millous (1929) reported an outbreak of 1,000 cases in Cochin-China in 1927, and Hargrave (1931) a similar epidemic in American Samoa during which 2,842 cases were observed in two months. All accounts, therefore, agree that the disease spreads with alarming

CHAPTER III

THE USE OF EARTHENWARE, PORCELAIN, ASBESTOS, AND GLASS FILTERS

ALTHOUGH at the present time collodion membrane filters have superseded others in the study of viruses, so much pioneer work has been done with the earthenware types that we feel a general account of their characteristics not to be out of place.

Types of Filter

The following different types of filter have been used in the study of virus diseases:

(*a*) diatomaceous earth or kieselguhr,
(*b*) unglazed porcelain,
(*c*) asbestos,
(*d*) and glass; their individual characters will now be described.

(*a*) Diatomaceous Earth Filters.

The Berkefeld. This type is made in Germany and is supplied in three grades according to the degree of permeability to water: these are the coarse 'Viel', medium 'Normal', and fine 'Wenig' filters, called the V, N, and W grades respectively. The V grade is the most commonly employed of the three, and should retain *B. prodigiosus* at a low pressure, such as 100–200 mm. of mercury, but if the pressure is raised above this limit, then not only *B. prodigiosus* but also *B. influenzae* will pass through (see Dible, 1932). The N type prevents the passage of bacteria, and the W grade retains the larger filterable viruses but allows the smaller forms to go through.

The physical characters of the Berkefeld candle have been investigated in detail by Kramer (1927) and Mudd (1922) and their findings will be mentioned later.

The Mandler. This American modification of the German Berkefeld filter is manufactured from kieselguhr, asbestos, and plaster of Paris, and is supplied in three sizes which are graduated according to the results of an air-pressure test after immersion in water. The 'preliminary' size resists a pressure of 2 to 5 lb. per square inch, the 'regular' 6 to 9 lb., and the 'fine' 10 to 16 lb. per square inch. In construction, the Mandler filter is more robust than the Berkefeld variety but has not been extensively used in the study of viruses.

The British Berkefeld filter is constructed differently from the above, is very fragile, and is made only in one grade which, according to our own observations, appears to possess about the same degree of porosity as the German Berkefeld V candle. Like the latter, the lower earthenware end of the bougie is cemented to a metal tube, and at this point fractures and leaks are prone to develop if the filter is roughly handled during cleaning.

(*b*) Porcelain Filters.

The Pasteur-Chamberland type is made in France and has been extensively used in virus research. It is constructed of unglazed porcelain consisting of a composition of kaolin and sand, and since the entire candle is made in a single piece free from joints, it possesses a mechanical

advantage over the Berkefeld type. The Pasteur-Chamberland filter also possesses the added virtue of being easily cleaned by heating the candle to a high temperature in an electric furnace.

Nine different grades of these filters are available, namely the L_1, L_1 *bis*, L_2, L_3, L_5, L_7, L_9, L_{11}, and L_{13} sizes. The L_1 is the coarsest filter of the range and the makers state that it is only intended for clarifying purposes, whereas the L_1 *bis* and L_2 candles retain the larger bacteria but allow the smaller micro-organisms such as, for example, the organism of bovine pleuropneumonia to pass through. An excellent summary of the general characters of the Pasteur-Chamberland range of filters is given by Dible (1932).

Galloway and Elford (1931), working with foot-and-mouth disease virus, adversely criticized the value of the Pasteur-Chamberland filters and stated that they found them to be extremely unreliable.

(*c*) Asbestos Filters.

The Seitz. Several sizes are made, the smallest of which is the most convenient for virus work, and consists of an asbestos disk clamped (at its circumference) between two metal surfaces. After each experiment, the disk is discarded and replaced by a fresh one (which can be sterilized by autoclaving). Two grades are supplied, namely the K (coarse) grade, intended for clarification, and the E K (fine) for retaining large bacteria and even small organisms such as *B. prodigiosus* or *Br. melitensis*, provided that the pressure is not raised above 100 mm. of mercury. We have used the E K grade and found that the viruses of vaccinia, ectromelia, Shope's rabbit fibroma, infectious myxomatosis of rabbits, and bacteriophage readily pass through. The permeability of the Seitz filter can be decreased by inserting two disks into the metal mantle; this should be done if at any time difficulty is experienced in withholding bacteria.

(*d*) Glass Filters.

The frittered glass variety is constructed entirely of glass and possesses certain advantages, but such filters have not been widely used for virus work owing to the ease with which they become clogged. A wide range of average pore sizes is available and the coarsest grade, which is only used as a clarifying filter for removing large tissue particles before attempting to pass the fluid through a finer filter, is perhaps the most useful of the whole range. The efficacy of the filter for clarifying may be enhanced by spreading a layer of asbestos pulp (made by shredding a Seitz disk and soaking it in saline) over the glass filter-bed surface before adding the material.

The finer grades of frittered glass filters are made in a great range of pore sizes and any dimension varying from 250 μ to 0·15 μ can be constructed to order. The manufacturers[1] supply data with each filter, stating the diameter of the largest pore, the average pore, and the filtration speed in c.c. of water per minute. We have tried one model possessing an average pore diameter of 0·667 μ, the largest pore being 0·91 μ, and with a filtration speed of 50 c.c. of water in 30 minutes, 1 second, for studies on myxoma virus and found it satisfactory. The only disadvantage we have experienced with these filters is that the glass pores soon become clogged unless the material is well diluted before attempting to filter.

The Cleaning of Filter Candles

New candles should be thoroughly washed in a stream of running water, placed in 1 : 1000 hydrochloric acid for some hours, then rewashed

[1] Schott & Gen, Jena, Germany.

before use; candles containing infective material can be disinfected by immersing them for 2 hours in a 2 per cent. phenol solution.

The Berkefeld type should be cleaned, immediately after use, by forcing a current of water in the reverse direction through the filter pores. After washing away most of the large particles which clog the pores of the candle, it should be immersed in a bowl of water containing a 2 per cent. solution of 'liquor trypsini co.' (B.P.) and left for 24 hours, preferably in an incubator at 37° C. The candle should next be thoroughly washed, scrubbed with a brush, tested for patency, dried, wrapped up in Kraft brown paper, and resterilized by autoclaving at 115° C. at 30 lb. per sq. in. for 1 hour.

The Pasteur-Chamberland variety can be cleaned in the same manner, or by placing the candles in a cold electric furnace and gradually heating them to white-heat. After half an hour's heating the current is switched off and the candles allowed to cool slowly.

Frittered glass type. Before using a new filter, it should be cleaned by drawing water through its pores followed by hot hydrochloric acid and water again, to remove organic matter. The filters are sterilized by first moistening them and then placing them in a cold Koch steamer which should be gradually heated, and after one hour's exposure at 100° C. the steamer should be allowed to cool before removing the filter. After use, the filters can be cleaned by immersing them in hot sulphuric acid to which has been added a mixture of sodium nitrate and sodium perchlorate for a period of 24 hours. Sulphuric acid and sodium bichromate are not recommended for cleaning as they tend to affect the electrical charge of the glass pores (see Laug, 1934).

Some Theoretical Considerations concerning the Process of Filtration through Earthenware Candles

A variety of different factors determine the ability or inability of a particle of fixed size to traverse the pores of an earthenware filter, for example:

1. The amount of positive or negative pressure applied.
2. The length of time for which it is exerted.
3. The viscosity of the material filtered.
4. The pH of the fluid in which the tissue is suspended.
5. The number of infective elementary bodies in the suspension.
6. The average diameter of the elementary bodies or other infective particles present.
7. The electrical charge of the filter and of the material to be filtered.
8. The average pore diameter of the filter.

In view of the many factors involved, consideration must be paid to each of these before any virus can be definitely asserted to be filterable. Special care must also be taken to ensure that the filter candle is free from structural defects such as cracks or flaws.

The Pasteur-Chamberland jointless type of bougie filter is probably the most satisfactory of the earthenware varieties, but it unfortunately suffers from certain serious disadvantages. For example, when filtering feebly infective emulsions through such a candle, much of the infective material (elementary bodies) becomes adsorbed and retained in the pores of the candle. Thus the filtrate may be inactive, even though the average pore diameter of the filter may be many times greater than the size of the virus. These remarks are applicable to the Berkefeld candle and, perhaps to a lesser extent, the Seitz asbestos disk.

The adsorptive action of earthenware filter candles on viruses can be demonstrated by the following experiment: Prepare a series of five decimal dilutions of desiccated rabbit's testis, infected with vaccinia virus, in saline, ranging from 1:1,000 to 1:10,000,000, and inoculate 0·2 c.c. of each dilution into the shaved skin of a rabbit. Make up about 25 c.c. of a 1:100 dilution of the same tissue, pass it through a Berkefeld V filter; prepare a second series of decimal dilutions of the filtrate, ranging from 1:1,000 to 1:10,000,000, and inoculate 0·2 c.c. of each as previously into the skin of the rabbit, alongside the first series. Examine the animal daily and note the sequence in which vaccinial papules develop, and by comparing the skin reactions produced by each set of dilutions the loss of infectivity of the filtrate due to adsorption will become evident. Much will depend on the individual factors prevailing at the time of performing the experiment, but on one occasion we observed that, whereas 0·2 c.c. of a 1:1,000,000 dilution of (unfiltered) virus emulsion produced a reaction, the same suspension after filtration only reacted up to 1:100 dilution. In another experiment on Shope's rabbit fibroma virus, the filtrate proved to be completely inactive, although the elementary bodies are no larger than those of vaccinia virus. On the other hand, we have observed that ectromelia virus, which is the same size as vaccinia virus (Elford, 1933), traverses the Berkefeld filter with far greater ease. The question thus arises as to what are these differences attributable, and in discussing this problem, Galloway and Elford (1931) have suggested that the activity of a filtrate is governed by the relative number of elementary bodies present in the tissue emulsion before filtration. Thus the greater the number of elementary bodies in the suspension, the more infective the filtrate will be.

It is also probable that a second factor may be the virulence or pathogenicity of the individual virus particles for the species of animal inoculated. For example, the British domestic rabbit is highly susceptible to infection with rabbit myxoma virus, less susceptible to vaccinial infection, and comparatively resistant to Shope's fibroma virus, so that if a rabbit were to be inoculated with a filtrate of one of these agents then the result would depend on the number of elementary bodies in the filtrate. To quote a hypothetical instance, the minimum infective dose of Shope's fibroma virus may be 50 elementary bodies, that of vaccinia 10, whereas only 1 elementary body of myxoma virus may suffice to infect a rabbit. The inactive filtrates obtained on passing Shope's fibroma virus through an earthenware candle may, therefore, have been due partly to loss of elementary bodies during filtration, and partly to the natural resistance of the British domestic rabbit to infection with this virus. Before it is possible, then, to assess the results and relative significance of filtration experiments with earthenware filters, it is necessary to consider the susceptibility of the test animal to infection, in addition to taking into account the other factors enumerated.

The Berkefeld filter is made of diatomaceous earth or a compound of silicic acid and carries an electro-negative charge. Mudd (1922) has shown that the pores of the Berkefeld candle are the site of an electrical potential difference consisting of a Helmholtz double layer, in which the wall of the filter is negatively charged and the liquid within positively charged. When suspensions of tissue are passed through the pores of such a candle, positively charged particles contained in the material become adsorbed and retained by the filter. Consequently all filters constructed of siliceous material, sand, porcelain, powdered glass, colloidal silica, or diatomaceous earth will adsorb basic dyes but allow acid dyes to pass through.

Thus Victoria blue, which is a basic dye, will not pass the Berkefeld candle, but congo red, which is an acid dye, will do so (Kramer, 1927). If, however, the filter candle is constructed of calcium carbonate and magnesium oxide, or plaster of Paris possessing a positive charge, then Victoria blue will traverse it, whereas congo red will not do so. Should, however, the congo red be diluted and made acid (following which it turns blue), then it will pass the plaster of Paris filter but not the Berkefeld variety. Thus by reversing the electrical charge, Kramer succeeded in reversing the filterability of a dye. It was also found that if a filter was constructed of both plaster of Paris and diatomaceous earth, it acquired a neutral charge and permitted both positively and negatively charged dyes to pass its walls. The behaviour of dyes when subjected to filtration through earthenware candles is similar to that exhibited by positively or negatively charged colloids and viruses, and thus Kramer (1927) found that a *Staphylococcus* bacteriophage would pass through a Berkefeld filter but not through one constructed of plaster of Paris; the same applies to vaccinia, rabies, and tobacco mosaic viruses which are negatively charged. Likewise, diphtheria toxin behaves in a similar manner, but tetanus toxin, which is electro-positively charged, is adsorbed by the negatively charged Berkefeld filter, although each filter only adsorbs a small quantity of toxin. Apart from the influence exerted by the charge of the filter candle over the transit of a small particle through its pores, another factor which plays an important role is the reaction of the fluid in which the virus is suspended. Many interesting observations have been reported on this subject, and it has been shown that substances which are normally retained by a filter can be made to pass through it by preliminary treatment of the candle with oil, egg white, peptone, or heated serum (see Muir and Browning, 1908–9; Holderer, 1912). For example, according to Grinnell (1929), Victoria blue B which normally does not pass the Berkefeld filter can be induced to go through it if the dye is suspended in hormone broth. Results analogous to the behaviour of dyes and other substances have been reported in connexion with viruses. For example, Ward (1929) has shown that the most active filtrates of vaccinia virus were obtained after emulsifying the vaccinial pulp in hormone broth of pH 7·6, and Ward and Tang (1929) demonstrated that vaccinia and herpes 'encephalitis' virus were more easily filterable when emulsified in hormone broth than in normal saline. Bronfenbrenner (1927) discovered that, if particles carrying a bacteriophage were deposited on a Berkefeld filter, the phage could be washed through the candle with broth, but not with water.

Sawyer and Frobisher (1929) reported that yellow fever virus was able to pass through the Berkefeld N candle when suspended in broth, but not if the infected tissues were emulsified in physiological saline. Foot-and-mouth virus has also been shown to have an optimum filtration range lying between pH 6·4 and 8·75 (see Galloway and Elford, 1931).

These findings suggest that suspensions for filtration should be prepared in broth and made alkaline before attempting to filter them through earthenware candles. The opposite opinion to this has been expressed by Green and Eagles (1931), who filtered large quantities of vaccinia virus through Berkefeld V, N, and British Berkefeld filters, and found that the hormone broth was not superior to distilled water as a diluent. They also maintained that the pH of the virus material did not seem to be important within the range pH 6·4 to 8·4. Furthermore, the lining of filters with egg white did not, in their opinion, enhance filterability of the virus.

Practical Considerations

Owing to the loss of virus resulting from adsorption during filtration through earthenware candles, the material to be filtered should possess an initial high degree of infectivity. At the same time, the concentration of tissue protein should be low and any large tissue particles in the suspension, liable to clog the pores of the filter, should be eliminated by preliminary centrifugation at 3,000 r.p.m. for half an hour, followed by filtration through coarse asbestos pulp similar to that employed by Laidlaw *et al.* (see p. 77) in their studies on influenza virus. These workers freed emulsions of lung tissue from excess organic matter by first passing the extracts through sterile asbestos pulp spread over the surface of a coarse-grade frittered glass filter, after which the material was diluted in saline and filtered through a collodion membrane. When using Elford's membranes, only a very low pressure, e.g. from 20 to 50 cm. of mercury, is required for filtration; with earthenware or Seitz filters a higher pressure is demanded, and this may be applied as positive force on the surface of the tissue extract, or by creating a partial vacuum inside the filter flask. The latter is more often used and the 'negative pressure' applied should be as low as possible, free from fluctuation, and never exceed 30 cm. of mercury if possible. The time occupied in filtration, the volume filtered, the temperature of the room, and the prevailing atmospheric pressure should be recorded among the other experimental data.

Having removed the filtrate, at the conclusion of the experiment, the filter candle should be tested for flaws and structural defects. This is done by making a dilute suspension of *B. prodigiosus* (a loopful of a 24-hours' culture of the organism in 20 c.c. of saline solution) and adding it to the mantle. According to Grinnell (1929), an old culture of *B. prodigiosus* should be employed as a young culture sometimes fails to pass through a filter which is permeable to an older growth. The bacillary suspension is next filtered under the same conditions as the virus material, and after about 20 c.c. have passed through, the filtrate is tested for the presence of bacteria by adding 2 c.c. amounts to 6 tubes containing glucose broth which are incubated at 37° C. for a week; 2 c.c. should also be inoculated into Robertson's bullock heart medium (see Mackie and McCartney, 1938) and incubated at 37° C. for 3 weeks for evidence of anaerobic organisms. If growth is absent, the candle may be assumed to be free from defects. It is sometimes the practice to filter the virus suspension and *B. prodigiosus* simultaneously and this procedure can be adopted if desired; when filtering feebly infective material, it is advisable to avoid this method, since Levaditi and Nicolau (1923) have demonstrated that rabies, vaccinia and herpes viruses can be wholly adsorbed by larger bodies such as carbon particles.

The Interpretation of Results

If the filter candle retains *B. prodigiosus* and the filtrate proves pathogenic to a susceptible animal, the result may be regarded as evidence in favour of the filterable nature of the infective agent. The experiment should, however, be repeated, using another filter of the same type, and if similar results are obtained the virus may be concluded to be filterable. It is also advantageous to ascertain which filters the virus fails to pass, and accordingly candles of different porosity should be tested.

If the filtrate proves to be non-pathogenic on animal inoculation, the test must be repeated several times before abandoning the experiments, and the pressure should be increased as much as possible, short of allow-

ing the control organism (*B. prodigiosus*) to pass through the candle. The infectivity of the filtrate can be concentrated either by spinning it in a high speed centrifuge at 15,000 r.p.m. for one hour, or by diminishing the bulk of the fluid by evaporation from the frozen state.

Should all these measures fail to yield an infective filtrate, then one of two alternatives is possible: either (*a*) the infective agent is too large or else (*b*) it has been completely adsorbed by the pores of the filter.

In conclusion, it may be stated that when employing earthenware, porcelain, and similar types of filter, a positive result is of value provided that the filter has been proved to be efficient, but in the case of a negative result being obtained, it does not necessarily imply that the agent is not filterable, since adsorption effects produced by these filters are sometimes so powerful that certain viruses, e.g. trachoma, have the greatest difficulty in traversing them.

The introduction of Elford's collodion membrane filters has helped to overcome many of the difficulties referred to in the previous pages, and in recent years their value in the field of virus research has been accorded universal recognition. The collodion membrane filter occupies a class by itself, and is eminently suitable for the filtration of viruses, because, owing to its thinness, there is little loss of infectivity resulting from adsorption of virus in its pores, and the comparative rapidity with which filtration is accomplished renders clogging of its pores less liable to occur in comparison with earthenware candles. Furthermore, collodion membranes can be accurately graded in porosity according to their average pore diameter, so that the size of the virus can be measured from the filtration data. The properties of these filters will be discussed in the following chapter.

REFERENCES

See p. 43.

CHAPTER IV

ELFORD'S COLLODION MEMBRANE FILTERS

THE introduction of these filters has greatly advanced the state of our knowledge concerning the relative size of different filterable viruses, since they have, to a large extent, overcome the undesirable adsorptive effects associated with earthenware filters. The basic principle underlying their construction depends on utilization of the antagonistic action of amyl alcohol and acetone, which affects the rate of aggregation of nitro-cellulose particles during evaporation whilst the membrane is in process of formation. Thus, whereas individually amyl alcohol and acetone are solvents of nitro-cellulose, when present together they tend to coagulate the nitro-cellulose, and the alcohol and ether present maintain this gelling tendency. Elford's ultra-filters have been evolved from a series of systematic studies on the physical properties of collodion films, during which it was discovered that collodion films exhibited one of two different types of structure. Either the membrane showed a coarse microgel appearance, consisting of a microscopically visible structure, or alternatively a finer ultragel composition which could only be seen to be particulate matter when viewed under the high power magnification of an ultra-violet light microscope.

Acetic acid and collodion membranes tended to show both the microgel and ultragel construction and were consequently unsuitable for filtration experiments in view of their excessive variability in pore size. Alcohol-ether membranes, on the contrary, consisted of the fine ultragel formation, and being of greater uniformity in average pore size, proved more suitable as membranes for the filtration of viruses. Elford also investigated the conditions governing the formation of ultragel structure and found that the ultragel structure was consequent upon spontaneous gelation of the collodion prior to the washing process in water, which served to fix the already potentially existing structure. From this information, Elford was able to increase the state of aggregation in nitro-cellulose, whilst the system still retained its gelling properties. This was accomplished by incorporating non-volatile coagulants with the solvents in the collodion, so that, as the solvents evaporated and coagulation commenced, the nitro-cellulose became more aggregated. Thus, the individual particle size of the units composing the ultragel tended to become larger, and synchronously the interstices of the membrane became enlarged, thereby increasing the permeability of the filter. A number of non-solvents were tested in order to find the most suitable one for increasing the permeability of the collodion membranes, and water was found to be the best, yielding membranes of good tensile strength. The greater the quantity of water added, the more permeable became the filter, until finally a maximum volume of 5 per cent. of water produced complete coagulation of the nitro-cellulose, yielding comparatively large pores that were only capable of arresting *B. prodigiosus*. Decrease in the permeability of the membrane was, on the other hand, produced by the addition of small quantities of ethyl alcohol, methyl alcohol (synthetic), or acetic acid. Thus, by incorporation of solvents or non-solvents in the collodion system, a series of membranes was produced possessing a range of average pore diameters (A.P.D.) varying from A.P.D. 3 μ to A.P.D. 10 mμ. Such values of A.P.D. were obtained from observation of the rates of water-flow (R.F.W.) through the membranes and determination of the

specific water content (S.W.C.) of each particular grade of membrane (Elford and Ferry, 1935). The measurements of R.F.W. were carried out by placing a section of membrane between the two limbs of a specially designed U-tube apparatus. In earlier experiments (Elford, 1931) the maximum pore size (M.P.S.) was determined from measurement of the critical air-pressure (C.A.P.).

Notes on the Preparation of Membranes

Workers desirous of making their own membranes are advised to consult Elford's original publications on the subject before attempting to do so (see Elford, 1929, 1931, 1933; Elford, Grabar, and Ferry, 1935), as well as the work of Asheshov (1933) and Bechhold (1907, 1908, 1908 *a*). The following notes indicate the general principles on which they are prepared.

Apparatus.

The collodion is poured into a glass cell of special dimensions where it is allowed to evaporate. The cell may be either of the 20 or 40 cm. size. Each cell is constructed of two thick squares of polished plate glass that have been cemented together with egg white, and the upper of these has a circular hole, 20 (or 40) cm. in diameter and 1 cm. deep, bored into it. The glass cell should be placed perfectly horizontal, and its position should be verified with a spirit-level gauge. All apparatus and solutions should attain a uniform temperature before use, and should remain for 1 hour at 22·5° C. prior thereto. Points requiring care are that the volume of the collodion solution should be accurately measured (75 c.c. collodion for a 20 cm. diameter cell), gently poured, and the period of evaporation accurately timed (75 minutes). The degree of humidity of the atmosphere should also be controlled.

Reagents.

Nitro-cellulose—Necol collodion No. 365A/9[1] has been principally employed.

As solvents ethyl alcohol, methyl alcohol, ether, glacial acetic acid, acetone, and amyl alcohol are required. These should be chemically pure, according to Elford, Grabar, and Ferry (1935), but Bauer and Hughes (1935) state that reagents of analytical grade are satisfactory without the additional purification recommended by Elford (1931).

With regard to solutions, amyl alcohol and acetic acid are added by volume and the remainder should be prepared on a gravimetric basis. They should be thoroughly mixed by placing them in a mechanical shaker for 4 hours at laboratory temperature before being used. Every procedure should be uniform and constant throughout and only small quantities of material made up at a time.

Nomenclature.

Elford has introduced a notation in order to describe the composition of the different collodion systems employed. Thus, 'N*a*/40 (*x*:*y*)' means Necol collodion containing '*a*' c.c. of amyl alcohol per 40 gm. of Necol (stock), diluted by the addition of '*x*/10' of its own weight of alcohol and '*y*/10' of ether. Any further modification of such a system is denoted as a volume addition per 100 gm. Therefore, N*a*/40 (*x*:*y*)+1 per cent. acetic acid means that 1 per cent. of the acid has been added per 100 gm. of the denoted system.

[1] A standard ether-alcohol collodion of Nobel Chemical Finishes, Ltd., Slough.

The following data indicate the properties of membranes prepared from N*a*/40 (1 : 9) systems consisting of '*a*' c.c. of amyl alcohol added to 40 gm. of Necol solution, then diluted with its own weight of a mixture of alcohol and ether in the rates of 1 : 9 parts by weight.

Conditions.

These are indicated below (Elford, 1931).

System	*R.F.W.*	*A.P.S.*	*C.A.P.*	*Membrane thickness*	*Remarks*
		μ.	lb./sq. in.	mm.	
N10/40 (1:9)	11·5	0·80	30	0·31	
„ +0·5% H_2O	13·0	0·87	28	0·31	
„ +1·0% „	17·2	1·0	22	0·32	Conditions:
„ +2·0% „	78·0	2·15	12	0·32	Vol. collodion . 75 c.c.
„ +0·5% HAc	1·2	0·26	175	0·33	Cell diameter . 20 cm.
„ +1·0% „	0·17	0·10	>200	0·35	Temperature . 22·5° C.
„ +1·5% „	0·065	0·06	>200	0·36	Evaporation period 75 mins.
„ +5% MeOH	0·75	0·20	180	0·34	
„ +10% „	0·23	0·114	>200	0·40	
„ +20% „	0·13	0·088	>200	0·45	

From the above it will be observed that a series of membranes ranging from A.P.S. (or A.P.D.) 2·15 μ to 0·06 μ are obtainable.

Summary of Procedure.

N10/40 (1 : 9) parent collodion under the conditions stated yields a membrane ranging from an A.P.D. of 0·65 μ to 0·85 μ. To increase permeability, small amounts of water in quantities of 0·25 per cent. are added so that a maximum A.P.D. of 2 μ is reached when 2 per cent. of water has been added.

To decrease the permeability of the membrane, acetic acid is added and the concentration is increased by 0·5 per cent. each time so that when a total of 2 per cent. acetic acid is reached a membrane of A.P.D. 10 mμ results. The intermediate pore sizes are obtained by varying the evaporation period, and in this way a graded series of membranes ranging in A.P.D. from 3 μ to 10 mμ can be prepared.

Washing of Membranes.

This is done in sterile distilled water which is changed thrice daily for a period of 14 days. Alternatively, they may be washed for 4 days in distilled water, 2 days in freshly boiled 20 per cent. alcohol, and a further 4 days in distilled water.

Sterilization and Storage.

Membranes can be sterilized either by placing them in cold distilled water, followed by boiling for 1 to 3 hours, or, alternatively, by carrying out some of the later processes of preparation in an ultra-violet light chamber, after which they can be stored in sterile distilled water.

Determination of A.P.D.

Before use, the pore size of each new batch of membranes should be measured by ascertaining the rate of water-flow pressure (R.F.W.) and the specific water content (S.W.C.) of each particular grade of membrane. The following quotation from Elford's (1931) publication describes the method employed for measuring the rate of water-flow:

Rate of Flow of Water (R.F.W.).

'The time taken for a known volume of distilled water to flow through a membrane of known area and thickness is measured under standard conditions of temperature

and pressure. From the data then known it is possible to calculate the rate of flow of water through the particular membrane under certain arbitrarily fixed conditions. By definition

$$\text{R.F.W.} = \text{c.c./sq. cm./min. for} \begin{cases} 0{\cdot}1 \text{ mm. membrane thickness.} \\ 100 \text{ cm. water pressure.} \\ 20^\circ \text{ C. temperature.} \end{cases}$$

Formula for calculation:

$$\text{R.F.W.} = \frac{Vt60 \times 1000}{\pi r^2 TP}, \text{ where } \begin{aligned} V &= \text{vol. water in c.c.} \\ t &= \text{membrane thickness in mm.} \\ r &= \text{membrane radius in cm.} \\ T &= \text{time of flow of vol. } V \text{ in sec.} \\ P &= \text{water pressure producing flow in cm.} \end{aligned}$$

$$= K\frac{Vt}{T}, \text{ where } K \text{ is constant for standard conditions of measurement.'}$$

Fig. 13 shows the form of cell used for these measurements.

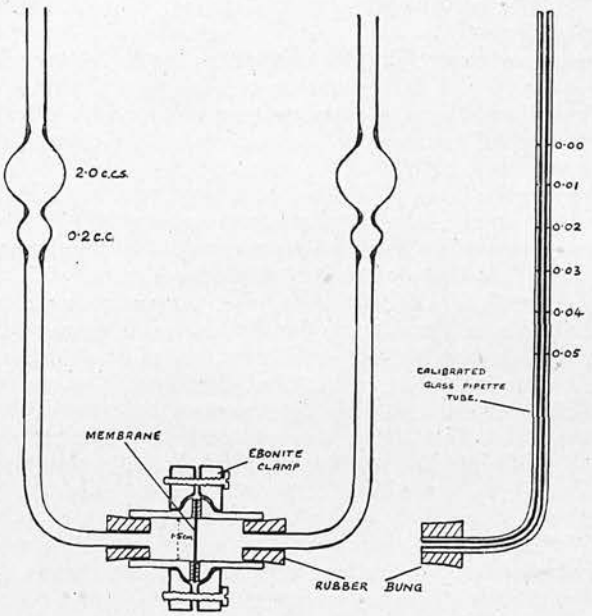


FIG. 13. Shows construction of simple cell employed by Elford (1931) for estimating rate of water-flow. It can be conveniently made by using two glass bottle necks suitably ground with carborundum. (Reproduced from Elford, 1931.)

For details regarding the estimation of S.W.C. see Elford and Ferry (1935), also Asheshov (1933).

Alternative Method

Elford's work has been successfully repeated by Bauer and Hughes (1935), working in America, who used a collodion product labelled Collodion X660–18.[1] These workers also prepared stock collodion solution, using Du Pont's nitro-cellulose parlodion sheets, the exact composition of which was as follows:

Parlodion shreds, 150 gm.; absolute alcohol, 250 gm.; anhydrous ether, 750 gm.; acetone, 1,150 gm.; and primary amyl alcohol, 575 c.c.

Before use, parlodion sheets were well washed six times in distilled water, twice in 95 per cent. alcohol, and twice in absolute alcohol, then

[1] Manufactured by Du Pont De Nemours of Parlin, New Jersey.

were used without drying. After the final washing, 250 gm. (316 c.c.) of absolute alcohol, which was freshly prepared by drying 95 per cent. alcohol over lime and distilling, was added to the parlodion shreds which were allowed to swell overnight. On the following day, 750 gm. (1,043 c.c.) of anhydrous ether was added and the mixture shaken at intervals until the parlodion was dissolved. The solution was diluted with its own weight of acetone (1,453 c.c.) and agitated in a mechanical shaker for 2 hours. Primary amyl alcohol was then added in the proportions of 10 c.c. to each 40 gm. of the mixture and the solution again shaken for 2 hours. Finally, the stock preparation was allowed to stand for 2 to 3 weeks before being used. Bauer and Hughes stated that membranes manufactured with such parent collodion gave satisfactory results, and they also drew attention to the fact that the percentage of acetone and amyl alcohol used by them was not the same as that recommended by Elford (1931).

To prepare membranes: the stock solution of collodion was diluted with an equal volume of a diluting mixture consisting of 1 part by weight of absolute alcohol and 9 parts of anhydrous ether. This diluted solution was measured in 200 c.c. amounts into a series of 6-ounce bottles fitted with screw caps lined with tin-foil.

Membranes made from such a solution possessed an A.P.D. of 0·6 to 0·8 μ, depending on the humidity and temperature at which evaporation of the solvents occurred. To make more permeable membranes, 2 c.c. of water was added to 200 c.c. of the diluted collodion and this yielded membranes of an A.P.D. 1·0 to 1·2 μ. For preparing membranes below 0·5 μ in A.P.D., glacial acetic acid of analytical quality was added, varying from 0·2 to 3·6 c.c. per 200 c.c. of diluted collodion solution. So many individual factors tended to affect the porosity of the membrane that Bauer and Hughes stated that no definite standards could be laid down, but in general they found that the addition of 1 c.c. of glacial acetic acid to 200 c.c. of collodion solution reduced the A.P.D. to 200 mμ, 2 c.c. reduced it to 100 mμ, and the addition of 3 c.c. reduced the A.P.D. still further to 15 mμ.

Further details were as follows: After addition of the acetic acid or water to the collodion, the mixture was shaken for 2 hours, the solution carefully poured into the cell, the contents of one bottle (200 c.c.) being used for a 40 cm. diameter cell. The optimum evaporation time was found to be 75 minutes, the temperature during evaporation 22° to 24° C., and the relative humidity from 60 to 65 per cent. After evaporation, to remove the membrane, the cell was immersed in water and, without exerting manual traction, the membrane permitted to free itself. The membranes, which were separated from each other by filter paper, were then washed in distilled water for 2 to 3 weeks and placed in a flat photographic developing tray. Disks were cut from the membranes and stored in distilled water in air-tight jars: they were calibrated before use according to the method recommended by Elford (1931).

Types of Filter Suitable and Pressure Applied

In a personal communication, Dr. Elford has informed us that two varieties of instrument are made, the one for positive and the other for negative pressure. Two sizes of each type are available, the larger holding 30 c.c., taking a 4 cm. membrane, and the smaller which has a capacity of 10 c.c. and is fitted with a 3 cm. membrane. Either positive or negative pressure can be used, but when preparing stock filtrates of small amounts less than 20 c.c., or when employing relatively 'tight membranes', it is

usually more convenient to apply positive force. Thus to prepare a bacteria-free filtrate of a pathological fluid (well-diluted and clarified), a membrane of A.P.D. 700 to 750 mμ should be used, with either a positive or a negative pressure of 25 cm. of mercury.

The Interpretation of the Results of Ultra-filtration

Like other forms of filter, the same factors govern the passage of particles through collodion membranes, but by virtue of their thinness and uniformity of porosity, many of the inherent disadvantages of earthenware filters have been eliminated. The properties of these membranes have been investigated with meticulous care by Elford (1933) who has inquired into the slight effects due to adsorption, variations in the thickness of individual membranes, and the influence of different pressures. After consideration of the mode by which each of these individual factors operate, Elford (1933) has been able to define the optimum conditions for filtration. For example, a 48-hours' old agar culture of *B. prodigiosus* suspended in broth at pH 7·6 was retained by all membranes of pore size less than 0·75 μ and, consequently, the average pore size of the limiting membrane furnished a good indication as to the size of the organism. It should be noted, however, that *B. prodigiosus* microscopically varies in size from 0·5 μ to 1·0 μ, so that even those forms which are 0·5 μ fail to pass through a membrane whose average pore size is 0·75 μ. The same effect was demonstrated in the case of the organism of pleuropneumonia, a 3-days' old serum broth culture at 37° C. containing particulate spherical bodies of 0·2 μ to 0·5 μ in size, together with invisible phases below this limit. By filtering the culture through membranes of graded porosity, it was shown that the larger visible spheres failed to pass membranes below 0·35 μ A.P.D., whereas the smaller forms did. A membrane of 0·35 μ therefore retained particles of 0·2 to 0·25 μ in size.

It was also pointed out that the concentration of bacteria in the initial bacillary suspension largely determined the number of them which entered the filtrate; an analogous observation was made in the case of foot-and-mouth disease virus by Galloway and Elford (1931). As filtration proceeds the pores of the membrane tend to become progressively clogged, until finally an end-point is reached at which the forces of adsorption and cohesion exert an abnormal effect and slow the rate of filtration. The filtration end-point may thus be experimentally ascertained and plotted graphically.

The results obtained with Elford's (1933) membranes showed that the first falling off in maximum filtrate concentration usually occurs at a pore size value varying from two to three times the absolute limiting value for non-filterability. Fig. 14 shows a graph indicating the filtration characteristics of *B. prodigiosus* and vaccinia virus when compared together.

The sizes of viruses as determined by ultra-filtration are in close agreement with those ascertained by ultra-violet light photomicrography. For example, ectromelia virus was estimated to measure 0·1 to 0·15 μ by filtration and 0·13 to 0·14 μ by micrometry, according to Barnard and Elford (1931). Likewise, Elford and Andrewes (1932) calculated that the elementary bodies of vaccinia measured 0·125 to 0·175 μ by filtration, and Barnard found them to be 0·17 to 0·18 μ by ultra-violet light photography.

Estimation of Particle Size.

From the results of filtration end-point tests obtained under the optimum experimental conditions, Table II indicates the probable size of the smallest particle just retained by a given membrane.

Table II

Membrane Average pore diameter	*Size of particle retained*
mμ	
10–100	(0·33–0·5) *d*
100–500	(0·5–0·75) *d*
500–1,000	(0·75–1·0) *d*

d = the average pore diameter of limiting membrane for optimum filtration conditions.

(Additional details regarding the construction of collodion membranes are dealt with by Elford, Grabar, and Ferry, 1935.) However, Elford

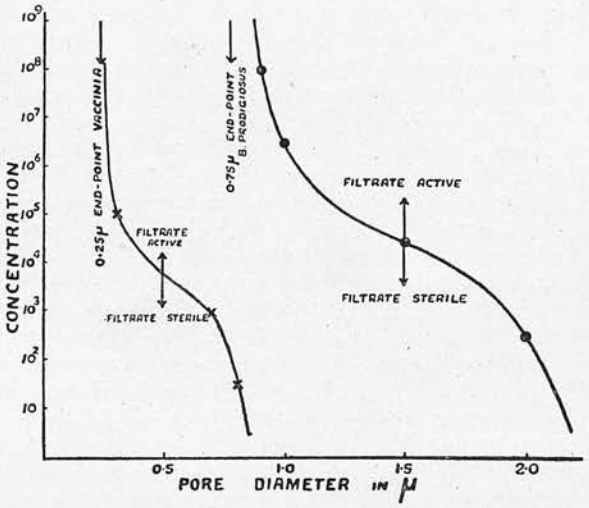


FIG. 14. This graph shows the behaviour of vaccinia virus and *B. prodigiosus* when subjected to ultra-filtration, and is reproduced from the work of Elford and Andrewes (1932).

(1933) has drawn attention to the fact that the number of particles (e.g. *B. prodigiosus*) present in a filtrate are dependent upon their concentration in the initial suspension, and Galloway and Elford (1931) have also referred to the same point in connexion with foot-and-mouth disease virus. This criticism of the value of ultra-filtration as a method for measuring the size of viruses has also been brought forward by Levaditi *et al.* (1936), who drew attention to certain pitfalls in the use of ultra-filtration methods. Working with lymphogranuloma inguinale virus, they obtained different sizes for the virus by employing tissue suspensions of varying degrees of pathogenicity. Thus, from filtering a feebly active tissue extract they calculated its size to be 600 to 800 mμ, but by using a more active suspension they found it to be 250 to 350 mμ, and by employing a highly virulent preparation they estimated the size of the virus to measure 100 to 140 mμ. From these observations, it was concluded that the more actively pathogenic was the virus material, the smaller appeared to be its size.

Levaditi *et al.* (1936) also stressed a second factor liable to influence the results, namely the susceptibility of the species of animal inoculated with the filtrate when the latter is tested for pathogenicity. For example,

when filtrates of lymphogranuloma inguinale tissue extracts were injected into a relatively insusceptible animal such as a mouse, they found that the virus appeared to measure 430 to 507 mμ; but if a more susceptible animal such as a monkey was used, then it was found that the virus measured 120 to 180 mμ in size.

Similar deductions were made earlier by Levaditi and Nicolau (1923) in connexion with the filtration of neurotropic viruses through collodion sacs. The French workers have accordingly concluded that the two factors mentioned have an important bearing on the problem and should be taken into account when measuring the size of viruses by ultra-filtration methods.

These observations are of great interest and suggest that further work on this aspect of ultra-filtration studies is necessary.

REFERENCES

Asheshov, I. (1933), *J. Bact.*, **25**, 323, 339.
Bauer, J. H., and Hughes, T. P. (1935), *J. gen. Physiol.*, **18**, 143.
Bechhold, H. (1907), *Hoppe-Seyl. Z.*, **60**, 257.
—— (1908), *Hoppe-Seyl. Z.*, **64**, 328.
—— (1908*a*), *Biochem. Z.*, **6**, 379.
Bronfenbrenner, J. (1927), *J. exp. Med.*, **45**, 873.
Dible, J. H. (1932), *Recent Advances in Bacteriology*, Churchill, London.
Elford, W. J. (1929), *Brit. J. exp. Path.*, **10**, 126.
—— (1931), *J. Path. Bact.*, **34**, 505.
—— (1933), *Proc. roy. Soc.*, B, **112**, 384.
Elford, W. J., and Andrewes, C. H. (1932), *Brit. J. exp. Path.*, **13**, 36.
Elford, W. J., and Ferry, J. D. (1935), *Brit. J. exp. Path.*, **16**, 1.
Elford, W. J., Grabar, P., and Ferry, J. D. (1935), *Brit. J. exp. Path.*, **16**, 583.
Galloway, I. A., and Elford, W. J. (1931), *Brit. J. exp. Path.*, **12**, 407.
Green, A. B., and Eagles, G. H. (1931), *Brit. J. exp. Path.*, **12**, 202.
Grinnell, F. B. (1929), *J. Bact.*, **18**, 175.
Holderer, M. (1912), *C.R. Acad. Sci., Paris*, **155**, 318.
Kramer, S. P. (1927), *J. infect. Dis.*, **40**, 343.
Laug, E. P. (1934), *Ind. Eng. Chem., Analyt. Ed.*, **6**, 111.
Levaditi, C., and Nicolau, S. (1923), *C.R. Soc. Biol., Paris*, **88**, 66.
—— —— (1923), *C.R. Acad. Sci., Paris*, **175**, 717.
Levaditi, C., Païc, M., and Krassnoff, D. (1936), *C.R. Soc. Biol., Paris*, **123**, 1048.
Mackie, T. J., and McCartney, J. E. (1938), *A Handbook of Practical Bacteriology*, Livingstone, Edinburgh.
Mudd, S. (1922), *Amer. J. Physiol.*, **63**, 429.
Muir, R., and Browning, C. H. (1908–9), *J. Path. Bact.*, **13**, 232.
Sawyer, W. A., and Frobisher, M. (1929), *J. exp. Med.*, **50**, 713.
Ward, H. K. (1929), *J. exp. Med.*, **50**, 31.
Ward, H. K., and Tang, F. F. (1929), *J. exp. Med.*, **49**, 1.

CHAPTER V

THE CENTRIFUGALISATION OF ELEMENTARY BODIES

In principle, the act of centrifugalising elementary bodies is no different from that of spinning down bacteria in the ordinary way. A much higher speed is, however, usually demanded, for owing to the smaller size of elementary bodies a greater force is required to deposit them.

The researches of Ledingham (1931) demonstrated that the elementary bodies of fowl-pox (and vaccinia) could be deposited by centrifugalisation at 12,000 revolutions per minute (r.p.m.) for 1 hour, after which the supernatant fluid lost its infectivity and the deposited material consisted of innumerable elementary bodies. Suspensions of elementary bodies prepared in this manner were agglutinated by specific antisera, and thus their aetiological relationship to fowl-pox was proved beyond all doubt. Amies (1934) used the same technique for centrifugalising the elementary bodies of zoster and varicella; these were obtained by diluting vesicle fluid in 0·86 per cent. saline containing 2 per cent. sodium citrate, centrifugalising at low speed to throw down tissue cells, and thereafter spinning at 12,000 r.p.m. for 30 minutes to deposit the elementary bodies. Such an antigen consisting of a suspension of elementary bodies was used by Amies (1934) for agglutination tests with the sera of patients who had recovered from zoster and varicella infections respectively.

Both Ledingham and Amies used the bucket type of centrifuge, but other varieties of instrument have also been employed with equal success and some of these are referred to below.

TYPES OF CENTRIFUGE

The majority of workers have employed machines of the high-speed type, but Craigie (1932) has shown that they are not essential, since elementary bodies can be deposited at a comparatively low speed provided that the instrument is of the angle design. Moreover, Professor Tulloch has expressed the opinion (in a personal communication) that the sedimentation rate can be varied by adjusting the pH of the suspending fluid.

Centrifuges in common use at the present time may be divided into four classes, namely:

1. Those fitted with a *bucket type* of rotating head, driven by a high-speed electric motor such as in the Ecco[1] machine, or by a slower speed motor operating via a geared pulley[2] similar to the type employed by Ledingham (1931).

2. *The angle centrifuge.* This is fitted with a slow-speed motor but is, nevertheless, capable of depositing elementary bodies by virtue of the fact that the construction of the head and the narrowness of the flat glass tube employed compensate for the loss of speed.

3. *The Sharples centrifuge.* This consists of a cylinder rotating vertically in its long axis at 16,000 r.p.m. into which material is introduced, and which is propelled by an electric motor fitted with a geared belt drive.

4. *Air-driven instruments.* These are based on the principle devised by

[1] Collatz Ltd., Berlin.

[2] Baskerville & Lindsay, Manchester.

Centrifugation of the Elementary Bodies of Infectious Myxomatosis of the Rabbit.

Analogous to bacteria, elementary bodies are particulate structures which may be removed from infective material by centrifugalization at high speed. With the use of an ultra-centrifuge at the Bacteriology Department of the University of Edinburgh, it was shown possible to deposit the elementary bodies of myxoma virus, thus demonstrating conclusively for the first time, the aetiological relationship of the elementary bodies of myxoma to the disease.

Henriot and Huguenard (1925), being propelled by a current of compressed air which strikes the slots of an air turbine that can be made to revolve at 60,000 r.p.m.

1. Centrifuges fitted with the Bucket Type of Head and driven by a directly coupled or geared electric motor

The Ecco centrifuge (bucket type).

Both Elford (1936) and we ourselves (van Rooyen and Rhodes, 1937) have used such an instrument. This is fitted with a direct drive (d.c.) electric motor which revolves at 15,000 r.p.m. and carries four buckets which accommodate hard glass tubes holding 5 c.c. each. The rotating head is enclosed inside a metal bowl, so as to eliminate air resistance during running, and the interior of the metal casing is cooled by means of a copper jacket through which cold water circulates. Elford (1936) states that the motor attains 4,000 r.p.m. in under a minute and 10,000 r.p.m. in just over a minute. It also slows down quickly and stops within a minute when turning at 4,000 r.p.m. and in one and a half minutes from 10,000 r.p.m.

Measuring the speed of the centrifuge.

By placing a revolution counter or tachometer on the end of the revolving spindle and timing the results with a stop-watch, the speed of the instrument can be calculated. At a high speed such as 15,000 r.p.m. this method is probably not the best, for owing to unavoidable losses due to slip and other effects the results tend to be inaccurate. Elford (1936) has devised a clever optical contrivance for estimating accurately the speed of the motor, and his description of it is as follows:

'A cylindrical tubular extension was attached to the screw cap which fitted the spindle of the centrifuge, projecting about 2 cm. above the top of the metal case. A hole was drilled through this tube at right angles to its axis. Light from a pointolite lamp was gathered by a lens and directed as a narrow beam through the hole in the centrifuge extension tube on to the periphery of a toothed disk which could be rotated at a controlled speed. The teeth were viewed in a telescope set in alignment with the beam of light. Suppose the centrifuge was spinning at N r.p.m.; then as the speed of the rotating disk is increased, the *first* stationary image seen in the telescope will occur when a tooth is just replaced in its position by the one following in the time interval between successive axial passages of the light through the hole in the centrifuge extension. This condition is given by $2N = xS$, where x = the number of teeth and S = the speed of the disk in r.p.m. The speed of the disk was regulated by means of a potentiometer resistance in series with a small universal motor and was measured by counting the revolutions with the aid of a speedometer and stop-watch. In order to embrace the complete range of speeds up to 15,000 r.p.m. two disks each having 72 teeth were coupled by gears in the ratio 1:7·5.

'This method enables the speed to be measured without affecting the centrifuge in any way, and is therefore to be preferred to the use of a tachometer.'

2. Centrifuges fitted with the Angle Type of Head and driven by directly coupled slow-running electric motor

The ordinary laboratory type of instrument (Bolaget) has been employed by Craigie (1932). The success of this machine depends on the fact that flat glass tubes are used, which possess an internal width of 4 to 5 mm., and consequently the elementary bodies have only to traverse a short distance before they strike the lateral wall of the tube which is placed at an angle of 28° from the vertical. With such a centrifuge a speed of 3,500 r.p.m. for 60 minutes was found to be sufficient for depositing the Paschen bodies of vaccinia.

An angle centrifuge fitted with a high-speed motor giving a speed of 15,000 r.p.m. is also obtainable and better results may be expected with this instrument.

3. The Sharples Centrifuge

The closed bowl technique (Schlesinger, 1936).

This ingenious principle makes it possible to obtain a centrifugal force of 20,000 × gravity and provides a quick method of sedimenting viruses. To operate the instrument, the inner wall of the rotating cylinder is first coated with a thin layer of agar, which is allowed to solidify, and a small amount of virus suspension is placed inside the cylinder. The latter has an internal radius of 2 cm., is 20 cm. high, and revolves at 32,000 r.p.m. Thus when turning at high velocity, the fluid within becomes evenly distributed as a thin film over the surface of the agar, so that the virus particles have only a very short distance to traverse before they impinge upon and adhere to the surface of the agar.

The interior of the bowl is coated with agar by first warming the cylinder, introducing melted agar into it, and allowing it to solidify whilst the cylinder is in motion. For particles less than 20 mμ in diameter 2 per cent. agar is used, and for viruses less than 60 mμ in diameter 1 per cent. agar.

4. Air-driven Centrifuges

McIntosh and Selbie's centrifuge.

McIntosh and Selbie (1937) have constructed a high-speed instrument, based on the design of the Henriot-Huguenard (1925) machine, which has no metal bearings and superficially resembles a large humming-top. It is driven by a current of compressed air which impinges upon the bottom of its outer casing into which a number of slots are cut. The machine therefore revolves on a cushion of air, and any loss of speed arising from friction due to bearings is eliminated. Four small tubes measuring 2 by 0·5 cm. are carried in narrow recesses on the top of the rotor and a speed of 60,000 r.p.m. can be obtained. Fig. 15 indicates the constructional details of the apparatus.

The rotor (A) consists of an inverted duralumin cone of 7·6 cm. diameter at its base with a vertex angle of 110°. The top of the base (B) is flat and has four radial recesses cut into it capable of taking four small tubes 2 by 0·5 cm. in size. The tubes are held down during spinning by a flat duralumin disk (C) which is screwed (D) down on the top of the cone. At the foot of the under surface of the rotor a series of cuts or grooves are made and arranged, either in a radial or helicoidal manner, so as to assist the driving force of the air after the principle of an air turbine.

The stator (E). This is a hollow cone made of phosphor bronze provided with nine holes round its interior (F), through which compressed air is forced and allowed to impinge upon the grooves cut into the sides of the rotor cone in an upward and anticlockwise direction. The angle of the stator is 90° to 92°, and the air-holes or jets should be drilled down (F) about 2 cm. from the upper and inner margin of the cone, at an angle of 45° to the axis of the cone when viewed antero-posteriorly and vertically when viewed from the side. The running of the instrument is controlled by an adjustable valve (G), and at the conclusion of the experiment the spinning rotor is lifted off the stator with a metal fork.

Compression plant. To maintain a speed of 60,000 r.p.m., a compressed air plant[1] driven by a 4-h.p. electric motor, and delivering 20 cubic feet per minute at a pressure of 80 to 90 lb. per sq. inch, is required.

Device for estimating speed of centrifuge. McIntosh and Selbie (1937) have used a stroboscope for calculating the speed of their air-driven centrifuge. The method involves fitting up a neon lamp, possessing a frequency

[1] Manufactured by B.E.N. Patents Ltd., London.

of 100 cycles per second, that is worked by an electrically driven tuning-fork yielding a frequency of 6,000 cycles per minute.

A V-shaped mark is made in the revolving top of the centrifuge and the neon lamp is held above it while it spins. When the instrument reaches 6,000 r.p.m. the V-shaped mark appears stationary, and also at each subsequent multiple of 6,000 r.p.m. Thus a speed of 60,000 r.p.m. is reached after the V mark has become stationary ten times. Halves and

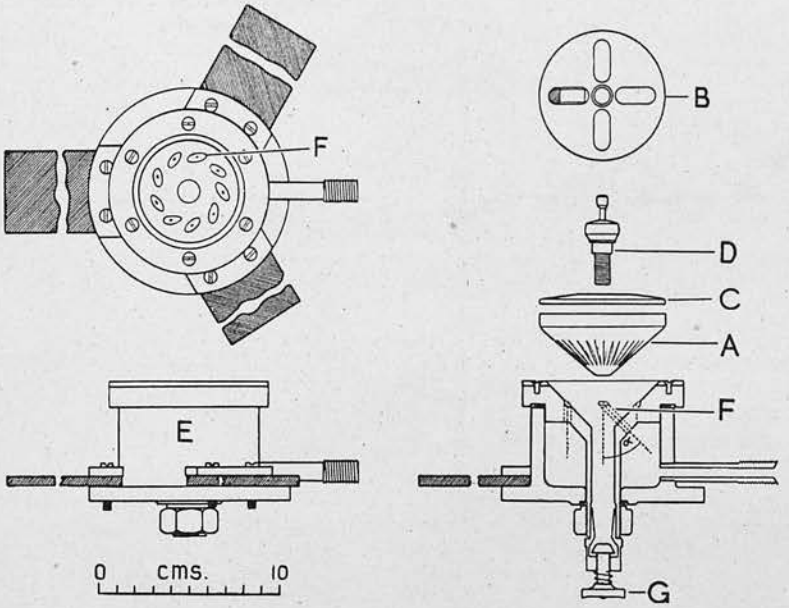


FIG. 15. Shows a sectional diagram illustrating the design of an air-driven centrifuge of the 'humming-top' type used by McIntosh and Selbie (1937). Reproduced from the *British Journal of Experimental Pathology*, vol. **18**, p. 162.

quarters of these figures can also be calculated, as two V's are visible at half a multiple and four at a quarter of a multiple.

The Svedberg centrifuge.

This is the fastest revolving type of centrifuge that has been constructed. The instrument is driven by oil turbines, and a speed of 160,000 r.p.m. has been obtained. The rotor weighs 12·5 gm., the cell 12·9 gm., and for sedimentation measurements up to 900,000 times gravity the two twin turbines operating at each end of the shaft are fed with oil at a pressure of 15 Kg./cm., and during rotation an atmosphere of 20 mm. of hydrogen is maintained. With such an instrument, Svedberg, Boestad, and Eriksson-Quensel (1934) have measured the sedimentation rate of dilute haemoglobin. So far, there are no reports in the literature of the machine being employed for the study of viruses, but an air-driven modification of Svedberg's instrument has been designed and used for the purpose, a description of which will now follow:

Biscoe, Pickels, and Wyckoff's (1936) modification of Svedberg's centrifuge.

These workers have designed an air-driven machine with which they have been able to measure optically the sedimentation rate of crystalline

proteins of the tobacco mosaic virus. The instrument embodies many of the principles incorporated in Svedberg's centrifuge, and in particular the optical device by which it is possible to watch the sedimentation rate of particles during centrifugalisation.

The rotor of the machine runs in a vacuum and is enclosed inside an air-tight metal casing. Two quartz glass windows are fitted opposite to each other at the top and bottom of this covering, and a beam of ultra-

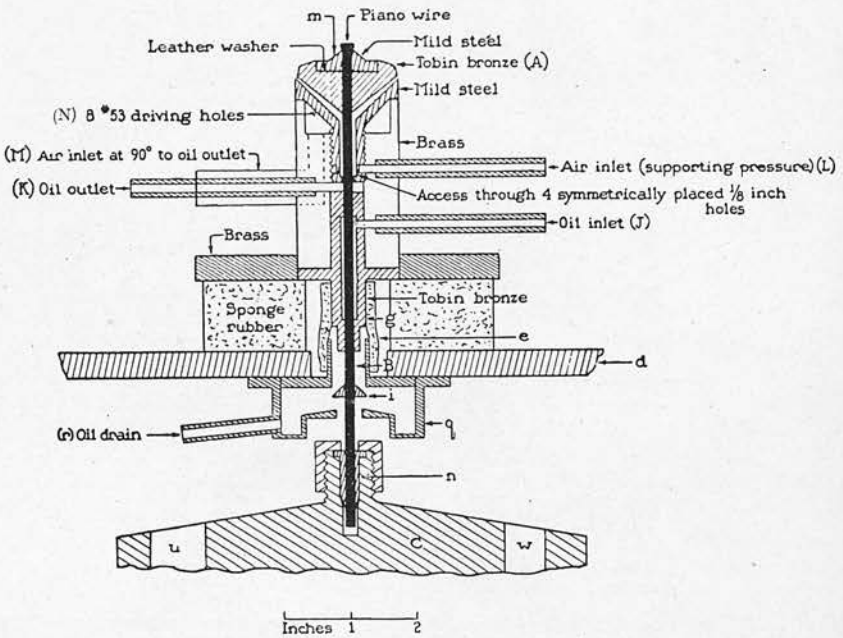


FIG. 16. Shows a cross-sectional drawing of the rotating system of the air-driven centrifuge designed by Biscoe, Pickels, and Wyckoff (1936). Reproduced from the *Journal of Experimental Medicine*, vol. **66**, p. 39.

violet light is passed through these so that the progress of sedimentation may be recorded photographically from time to time. For details regarding the fitting of the optical equipment, the reader is referred to the work of Svedberg (1934), Svedberg and Nichols (1927), and Svedberg, Boestad, and Eriksson-Quensel (1934).

Fig. 16 indicates the propelling mechanism of the Biscoe-Pickels-Wyckoff centrifuge seen in cross-section, and its operation is as follows: A current of air under pressure enters at either port (L) or (M), passes upwards and impinges against grooves cut into the sides of a mobile bronze turbine (A) so that this is set in motion. The latter is attached to the rotor (C) of the centrifuge by means of a flexible shaft (B) which revolves simultaneously. Additional constructional details are as follows:

(A). Bronze air-driven turbine, the conical surfaces of which have grooves cut into them. For details regarding the dimensions, number, and position of these milled flutings see Beams and Pickels (1935).

m. Leather washer acting as a turbine clutch.

(B). A steel shaft of piano wire 0·100 to 0·110 in. in diameter capable of withstanding 300 to 900 revolutions per second (r.p.s.). For speeds less than 300 r.p.s. a piece of 3/16 in. drill rod should be used instead, in order to eliminate vibration.

(N). A mild steel conical plate containing a number of holes acting as ports

through which air is forced from inlet (M) at 100 lb. per sq. in., requisite for a speed of 900 r.p.s. (see Beams and Pickels, 1935).
(L) is the air inlet referred to as the passage for introducing (supporting pressure) air at 30 to 40 lb. per sq. in. used for slow running at 200 r.p.s.
(K) and (J) are inlets for oil supplied at a pressure of 5 to 15 lb. per sq. in.
g. Oil-seal bearing.
q. Oil-well.
(r). Oil-drain.
i. Conical deflector for oil supply.
e. A short length of rubber pressure tubing.
n. Rotor clutch.
c. Rotor made of duralumin alloy 14 S.T., 7 in. in diameter, of tapering thickness, being 1 in. at periphery and 2 in. at centre. The outside edge of the cell hole is $\frac{1}{2}$ in. from the periphery of the rotor, and the distance between the centre of the cell (w) and axis of rotation is 6·5 cm.
w. Cell for holding suspension to be centrifuged. Is similar to that of Svedberg's. Its main difference is that, being filled from the end during assembly, an oil-seal is unnecessary.
u. Dummy cell for balancing rotor.
d. is the top of the centrifuge housing made of $\frac{1}{2}$ in. boiler plate into which the upper of the two quartz glass windows is fitted. This is not shown in the sectional diagram. The remainder of the rotor is housed in an air-tight casing constructed of chrom-vanadium steel, and while it revolves a vacuum of less than 1 mm. of mercury is maintained in the chamber by a quick-operating oil pump.

The optical system used for estimating the rate of sedimentation of solids within the cell during centrifugalisation is constructed as follows: Light rays from a mercury vapour arc lamp are rendered parallel through quartz condensing lenses, passed through bromine and chlorine filters, reflected by an aluminium mirror through quartz glass windows placed in the bottom of the vacuum chamber, through the cell (every time the apertures register during rotation), through the top quartz window fitted in (d), reflected by a second aluminium mirror, and finally focused by a 100 cm. focal length quartz-fluorite lens upon a photographic plate on which the image is recorded. The total distance from the centre of the cell to the photographic plate is 4·5 metres. Exposures are made during running of the centrifuge by means of an electromagnetically operated shutter placed between the collecting lens and the filter; the exposures vary from 1 to 10 seconds according to the sensitivity of the photographic plate employed.

Speed. The speed of the rotor is measured with a stroboscope. The method used consists of watching a spot placed on the top of the turbine (A) through a slotted disk that is mounted, together with a small magneto, on the shaft of an electric motor. The speed of the centrifuge is thus calculated directly in terms of the voltage developed by the magneto. At 900 r.p.s. (or 60×900 r.p.m.) the centrifugal force at the centre of the cell is estimated to be 225,000 times gravity, and at 800 r.p.s. (or 48,000 r.p.m.) the force is 180,000 times gravity.

Heating during running. After some hours of continuous use, the maximum rise of temperature for 800 r.p.s. is found to be 2° C.

The Size of Viruses Demonstrated by Centrifugalisation

The centrifuge designed by Svedberg was fitted with specially constructed windows which allowed optical observations to be made during centrifugation and thus record the rate of sedimentation. Bechhold and Schlesinger (1931) employed the ordinary bucket type of high-speed centrifuge for similar studies, and later Schlesinger (1932) used the same method for calculating the size of viruses and bacteriophages. In his experiments, Schlesinger centrifuged the virus suspension in a wide

flat-bottomed tube 5 cm. long and 1 cm. broad, containing at its foot a thick disk of filter paper the purpose of which was to minimize dispersion of the deposited virus particles after centrifugalisation. The infectivity-titre of the fluid was first ascertained before centrifugalisation (C_0) and later compared with the loss in titre after centrifugalisation (C_t), from which he was able to deduce the probable size of the virus particles (d) by application of the following formula:

$$d = 6{\cdot}15\times10^8\sqrt{\left(\frac{\eta h \log C_0\times C_t}{(\sigma_p-\sigma_m)RTN^2}\right)},$$

where d = the diameter of the particles in mμ, η = the viscosity of the medium in C.G.S. units, σ_p = the density of the particle, σ_m the density of the medium, h = the height of the column of liquid, R = the distance of the filter paper from the axis of rotation, N = the r.p.m. of the centrifuge, and T = the time of centrifugalisation in minutes. The above formula has been based on a series of experiments made by Schlesinger (1932) on the size of a range of gold sols of known dimensions.

The specific gravity of a virus is best calculated by centrifuging suspensions made up in varying densities of saccharose solution, to determine the lowest concentration of sugar solution in which the virus fails to be deposited. For example, Schlesinger and Andrewes (1937), using the Sharples centrifuge, found that after spinning Shope's fibroma virus for 75 minutes at 33,000 r.p.m., there was just detectable deposition in a medium of specific gravity 1·25. They therefore assumed that the specific gravity of the virus was 1·3, and from the results of their calculations concluded that the fibroma virus measured 126 to 141 mμ, and the papilloma virus 32 to 50 mμ. These figures closely approximated to those obtained by ultra-filtration, from which it was learned that the fibroma virus measured 125 to 175 mμ, and the papilloma agent 23 to 35 mμ in diameter.

Although the sizes of viruses and bacteria measured by Schlesinger's technique were found to be in good agreement, they invariably appeared to be about 30 per cent. higher than the values obtained by ultra-violet light and ultra-filtration, and accordingly Elford (1936) reinvestigated the problem and introduced the inverted capillary tube technique which overcame certain disadvantages associated with Schlesinger's (1932) technique.

Elford's (1936) improved method greatly facilitated the obtaining of samples of material after centrifugalisation, and minimized the possibility of redispersal of the virus particles after their deposition. It also enabled the investigator to compare the difference in infectivity of the fluid within the cell before and after centrifugalisation, from the results of which it was possible to calculate the size of the virus particles (d) by direct application of Stokes's law, the following formula being used:

$$d = 7{\cdot}94\times10^7\sqrt{\left(\frac{\eta\log\dfrac{x_1+l}{x_1+lC_t/C_0}}{(\sigma_p-\sigma_m)N^2t}\right)},$$

where d = the diameter of the particle in mμ, x_1 = the distance of the top of the capillary from the centre of rotation (= 7·25 cm. for metal cell or 6·75 cm. for glass cell), l = depth of inverted capillary tube (= 1 cm.), σ_p = the density of the particle, σ_m = the density of the medium, η = the viscosity of the medium, N = the speed of rotation in r.p.m., t = the time of centrifugalisation in minutes, C_0 = initial concentration, and

Nicht einzeln im Buchhandel. *Ueberreicht vom Verfasser.*

Sonderabdruck aus dem

Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten.

I. Abteilung. Originale.

1937. Bd. 140.

Verlag von Gustav Fischer in Jena.

Printed in Germany.

[From the Department of Bacteriology, Edinburgh University, Scotland.]

Centrifugation of the elementary bodies of infectious myxomatosis of the rabbit.

By **C. E. van Rooyen** and **A. J. Rhodes.**

Introduction.

In an earlier number of this journal one of us described the presence of elementary bodies in infectious myxomatosis of the rabbit (van Rooyen, 1937). These closely resemble the elementary bodies of vaccinia, fowl-pox, ectromelia, and other virus diseases, measuring from 0.31 μ to 0.36 μ in diameter when stained by Paschen's method, and sometimes showing chain-

in Table III was noted at this time. The supernatant fluid failed to give any reaction and appeared to be non-infective.

Table III.

Dilutions	Uncentrifuged washings	Reconstituted deposits	Dilutions	Supernatant
		Rabbit 5.		
1/1000	Typical myxomatous papule 4.5 cm diameter	Typical myxomatous papule 3 cm diameter	Undiluted	
			1/10	
1/10 000			1/100	No
1/100 000	No reaction	No reaction	1/1000	reaction
1/1 000 000			1/10 000	
1/10 000 000				

Summary.

When a series of rabbits was injected with uncentrifuged eye-washings (containing elementary bodies) typical myxomatous papules were produced. When these washings were centrifuged at high-speed the deposit was found to contain numerous elementary bodies, which could not be demonstrated in the supernatant fluid. This deposit, suspended in saline to constitute the original volume, was capable of producing typical myxomatous papules, whereas the supernatant fluid was non-infective.

These results indicate that the aetiological agent of infectious myxomatosis of the rabbit is the elementary body.

Conclusions.

1) The elementary bodies of infectious myxomatosis of the rabbit can be separated by high-speed centrifugation (15 000 r.p.m. for 2 hours).

2) On injection intradermally into rabbits the separated deposit containing these bodies produces typical myxomatous papules; the supernatant fluid is non-infective.

References.

Rivers, T. M., a. Ward, S. M., J. of exper. Med. **66**, 1 (1937). — van Rooyen, C. E. Zbl. Bakter. I Orig. **139**, 130 (1937).

C_t = the concentration after spinning for time t. For conditions such that $C_t/C_0 = 0{\cdot}1$ this relationship becomes:

$$d = k\sqrt{\left(\frac{\eta}{(\sigma_p - \sigma_m)N^2 t}\right)},$$

where $k = 1{\cdot}78 \times 10^7$ for the metal cells, or $1{\cdot}84 \times 10^7$ for glass cells.

By employing this principle for measurement, Elford and Andrewes (1936) found that vaccinia virus measured 170 to 180 mμ, influenza virus 87 to 99 mμ, and the Rous sarcoma virus 60 to 70 mμ.

Elford and Galloway (1937), using this inverted capillary tube technique, found that the size of foot-and-mouth virus was 20 mμ, assuming that the density of the particles was 1·30. Different strains of foot-and-mouth virus appeared to be of the same size and moreover their particles tended to be relatively uniform in dimension.

The size of vesicular stomatitis virus was also measured by the same technique, but its particle size tended to vary and ranged from 74 to 60 mμ according to whether the density of the particle was assumed to be 1·20 or 1·30 respectively. Subsequent studies by Tang, Elford, and Galloway (1937) showed that equine encephalomyelitis virus measured 39 to 32 mμ, louping-ill virus 27 to 22 mμ, and a *B. megatherium* bacteriophage 30 to 37 mμ. The need for more precise knowledge regarding the densities of the particles was emphasized by these workers.

Table III gives a tabulated account of the size of viruses estimated by different methods (see p. 53).

McIntosh and Selbie (1937) carried out similar methods of measurement by using their air-driven centrifuge constructed on the lines of the Henriot and Huguenard (1925) instrument, capable of attaining a speed of 60,000 revolutions per minute (see p. 46). The sedimentation rate of various bodies was expressed graphically to show 'a sedimentation angle', and the size of the particle was proved to be proportional to the square root of that angle, i.e. $D = K \times \tan\theta$, where D = the diameter of the particle, K = the speed of the centrifuge, and θ = the sedimentation angle.

These workers have used this simple formula for determining the size of virus particles, and have drawn attention to the similarity of their formula to that of Stokes. From their results, they estimated that vaccinia virus measured 99 to 220 mμ from the sedimentation angle, and 103 to 240 mμ from their modification of Stokes's equation.

REFERENCES

Amies, C. R. (1934), *Brit. J. exp. Path.*, **15**, 314.
Beams, J. W., and Pickels, E. G. (1935), *Rev. sci. Instrum.*, **6**, 299.
Bechhold, H., and Schlesinger, M. (1931), *Biochem. Z.*, **236**, 387.
Biscoe, J., Pickels, E. G., and Wyckoff, W. G. (1936), *J. exp. Med.*, **64**, 39.
Craigie, J. (1932), *Brit. J. exp. Path.*, **13**, 259.
Elford, W. J. (1936), *Brit. J. exp. Path.*, **17**, 399.
Elford, W. J., and Andrewes, C. H. (1936), *Brit. J. exp. Path.*, **17**, 422.
Elford, W. J., and Galloway, I. A. (1937), *Brit. J. exp. Path.*, **18**, 155.
Henriot, E., and Huguenard, E. (1925), *C.R. Acad. Sci., Paris*, **180**, 1389.
Ledingham, J. C. G. (1931), *Lancet*, **2**, 525.
McIntosh, J., and Selbie, F. R. (1937), *Brit. J. exp. Path.*, **18**, 162.
Schlesinger, M. (1932), *Z. Hyg. InfektKr.*, **114**, 161.
—— (1936), *Nature*, **138**, 549.
Schlesinger, M., and Andrewes, C. H. (1937), *J. Hyg., Camb.*, **37**, 521.
Svedberg, T. (1934), *Naturwissenschaften*, **22**, 225.
Svedberg, T., Boestad, G., and Eriksson-Quensel, I. B. (1934), *Nature*, **134**, 98.
Svedberg, T., and Nichols, J. B. (1927), *J. Amer. chem. Soc.*, **49**, 2920.
Tang, F. F., Elford, W. J., and Galloway, I. A. (1937), *Brit. J. exp. Path.*, **18**, 269.
van Rooyen, C. E., and Rhodes, A. J. (1937), *Zbl. Bakt.*, Abt. I, orig., **140**, 117.

CHAPTER VI

THE PARTICULATE NATURE OF VIRUSES—A SUMMARY OF RESULTS

MODERN research has suggested that filterable viruses are minute living agents, similar to the ordinary bacteria, of definite size, and capable of division and multiplication. In the preceding chapters, we have described various methods by which the size of viruses can be estimated; Table III contains a summary of these results which show that the viruses of different diseases vary considerably in their dimensions, some being as large as 275 mμ, others as small as 10 mμ. Table IV gives a list of viruses arranged in diminishing order of magnitude, among which have been included *B. prodigiosus* and the molecules of haemocyanin, serum albumin, pseudo-globulin, and egg albumin, in order to furnish comparative information. The data provided in Table III not only indicate the dimensions of individual viruses, but are also of interest because they prove the accuracy of different physical methods of measurement employed for the purpose. Thus, it will be observed that there is close agreement between the dimensions of the same virus when estimated by the application of physical principles so widely different as ultra-filtration and ultra-centrifugalisation, and ultra-violet light photography.

The Visible Viruses.

As early as 1887 Buist of Edinburgh (see Gordon, 1937; Mackie and van Rooyen, 1937) and later, Paschen (1906) of Hamburg, observed independently that the causal agent of vaccinia was a minute micro-organism that could be demonstrated in stained preparations under the ordinary microscope. In succeeding years other workers made similar observations in connexion with other virus diseases, and thus at the present time it is generally accepted that the designation 'ultramicroscopic and invisible viruses' is frequently a misnomer, for certain are capable of being stained and can be seen with the ordinary microscope. The limit of visibility attainable with the ordinary microscope has been fully discussed elsewhere (see Ch. I), and it only remains to repeat that any virus possessing a size of 67 mμ should (according to Coles, 1929) be visible as a stained particle under the ordinary microscope. Under dark-ground illumination the limits of resolution are greatly enhanced, and it should therefore be possible to see particles even smaller than this—a fact which has recently been supported by the careful observations of Merling-Eisenberg (1938), who succeeded in demonstrating phage particles measuring 25 mμ in size by the use of dark-ground illumination and an optical system possessing a numerical aperture of 1·25.

The following is a list of sixteen viruses which have been stained and demonstrated under the microscope in the form of elementary bodies: canary-pox, mouse ectromelia, fowl-pox, herpes, inclusion conjunctivitis, infectious myxomatosis of rabbits, Japanese encephalitis, lymphogranuloma inguinale, molluscum contagiosum, morbilli, psittacosis, rabbit fibroma of Shope, trachoma, variola-vaccinia, varicella, and zoster. References to literature regarding the elementary bodies found in these diseases are contained in Table III.

TABLE III.

Name of disease	Size of virus in mμ estimated by: ultra-filtration	centri-fugalisation	ultra-violet light micro-scope	Authority	Elementary bodies demonstrated in stained film preparations under the ordinary microscope by
Aujeszky's disease ('mad itch', pseudo-rabies, or infectious bulbar paralysis)	100–150	..	..	Elford and Galloway (1936)	..
Borna disease of horses	85–125	..	..	Elford and Galloway (1933)	..
Canary-pox	125–175	..	125–175	Burnet and Barnard (1933)	Herzberg (1936).
Ectromelia	100–150	..	130–140	Barnard and Elford (1931)	Marchal (1930); Paschen (1936).
Equine encephalomyelitis	20–30	..	..	Bauer, Cox, and Olitsky (1935)	..
,, ,,	20–35	32–39	..	Tang, Elford, and Galloway (1937)	..
Foot-and-mouth disease	8–12	..	..	Galloway and Elford (1931)	..
,, ,,	8–12	..	..	Krassnoff and Reinié (1937)	..
,, ,,	..	17–20	..	Elford and Galloway (1937)	..
Fowl plague	60–90	..	..	Elford and Todd (1933)	..
Fowl-pox	..	..	..	..	Borrel (1904); Woodruff and Goodpasture (1929, 1930); Ledingham (1931).
Herpes	100–150	..	..	Elford, Perdrau, and Smith (1933)	Taniguchi *et al.* (1934); Amies (1934).
,,	100–150	..	..	Païc, Krassnoff, and Reinié (1938)	Herzberg (1936).
Inclusion conjunctivitis	233	..	..	Thygeson (1934 *a*)	Lindner (1909, 1910, 1911); Thygeson and Mengert (1936).
,, ,,	153–206	..	..	Tilden and Gifford (1936)	..
,, ,,	200	..	..	Julianelle, Harrison, and Lange (1938)	..
Infectious myxomatosis of the rabbit (Sanarelli)	..	..	..	..	Aragão (1911); van Rooyen (1937).
Influenza (porcine)	80–120	..	..	Elford, Andrewes, and Tang (1936)	..
,, (human: W.S. strain)	..	87–99	..	Elford and Andrewes (1936)	..
Japanese encephalitis	..	..	..	..	Taniguchi *et al.* (1935).
Lymphogranuloma inguinale	125–175	..	..	Miyagawa *et al.* (1935)	Miyagawa *et al.* (1935 *a*).
,, ,,	125–175	..	..	Broom and Findlay (1936)	Findlay, Mackenzie, and MacCallum (1938).
,, ,,	100–140	..	..	Levaditi, Païc, and Krassnoff (1936 *b*)	..
Louping-ill	15–20	..	..	Elford and Galloway (1933 *a*)	..
,, ,,	..	22–27	..	Tang, Elford, and Galloway (1937)	..
Lymphocytic choriomeningitis	100–150	..	..	Rivers and Scott (1936)	..
Molluscum contagiosum	..	..	..	..	Lipschütz (1911); von Prowazek (1911).
Morbilli	..	..	..	..	Taniguchi *et al.* (1935 *a*).
Newcastle disease of fowls	80–120	..	..	Burnet and Ferry (1934)	..
Poliomyelitis	12–17	..	..	Theiler and Bauer (1934)	..

TABLE III—*continued*

Name of disease	*Size of virus in mμ estimated by*			*Authority*	*Elementary bodies demonstrated in stained film preparations under the ordinary microscope by*
	ultra-filtration	*centri-fugalisa-tion*	*ultra-violet light micro-scope*		
Poliomyelitis	8–12	..	..	Elford, Galloway, and Perdrau (1935)	..
,,	8–12	..	..	Kling, Païc, Krass-noff, and Haber (1938)	..
Psittacosis	220–330	..	..	Levinthal (1935)	Lillie (1930); Coles (1930); Levinthal (1930).
Rabies (fixed virus)	100–150	..	..	Galloway and Elford (1936)	..
,, ,, ,,	100–150	..	..	Yaoi, Kanazawa, and Sato (1936)	..
,, (street virus)	160–240	..	..	Païc, Krassnoff, Haber, Reinié, and Voet (1938)	..
Rabbit fibroma virus of Shope	125–175	126–141	..	Schlesinger and Andrewes (1937)	Paschen (1937).
Rabbit papilloma virus of Shope	23–35	32–50	..	Schlesinger and Andrewes (1937)	..
Rift Valley fever	23–35	..	..	Broom and Findlay (1933)	..
Rous sarcoma	75–100	..	..	Elford and Andrewes (1935)	..
,, ,,	75–100	..	..	Yaoi and Nakahara (1935)	..
,, ,,	..	60–70	..	Elford and Andrewes (1936)	..
St. Louis encepha-litis	22–33	..	..	Bauer, Fite, and Webster (1934)	..
,, ,,	20–30	..	..	Elford and Perdrau (1935)	..
Sand-fly fever	160	..	..	Shortt, Pandit, and Rao (1938)	..
Trachoma	200	..	..	Thygeson (1934); Thygeson and Proctor (1935)	Halberstaedter and von Prowazek (1907); Halberstaedter (1912).
Vaccinia	125–175	..	..	Elford and Andrewes (1932 *a*)	Buist (1887; see Gordon, 1937; and Mackie and van Rooyen, 1937).
,,	140–160	..	..	Levaditi, Païc, and Krassnoff (1936 *a*)	Paschen (1906, 1913); Ledingham (1931); Nauck and Paschen (1933).
,,	..	170–180	..	Elford and Andrewes (1936)	..
,,	..	103–240	..	McIntosh and Selbie (1937)	..
Varicella	..	..	..	..	Aragão (1911 *a*); Paschen (1917, 1933).
Vesicular stomatitis	70–100	..	..	Galloway and Elford (1933)	..
,, ,,	70–100	..	..	Bauer and Cox (1935)	..
,, ,,	60–90	..	..	Levaditi, Païc, Krass-noff, and Voet (1936)	..
,, ,,	..	60–74	..	Elford and Galloway (1937)	..
Yellow fever	18–27	..	..	Findlay and Broom (1933)	..
,, ,,	18–27	..	..	Bauer and Hughes (1934)	..
Zoster	..	..	..	..	Paschen (1933); Taniguchi *et al.* (1934); Amies (1934).

TABLE IV

Virus or other entity	*Size in mμ*	*Authority*
Bacillus prodigiosus	750	Elford (1936).
Psittacosis	275	Levinthal (1935).
Inclusion conjunctivitis	233	Thygeson (1934*a*).
Trachoma	200	Thygeson and Proctor (1935).
Sand-fly fever	160	Shortt, Pandit, and Rao (1938).
Canary-pox	150	Burnet and Barnard (1933).
Lymphogranuloma inguinale	150	Broom and Findlay (1936).
Rabbit fibroma of Shope	150	Schlesinger and Andrewes (1937).
Vaccinia	150	Elford and Andrewes (1932*a*).
Aujeszky's disease	125	Elford and Galloway (1936).
Ectromelia	125	Barnard and Elford (1931).
Herpes	125	Elford, Perdrau, and Smith (1933).
Lymphocytic choriomeningitis	125	Rivers and Scott (1936).
Rabies (fixed virus)	125	Galloway and Elford (1936).
Borna disease	105	Elford and Galloway (1933).
Influenza (porcine strain)	100	Elford, Andrewes, and Tang (1936).
Newcastle disease of fowls	100	Burnet and Ferry (1934).
Rous sarcoma	85	Elford and Andrewes (1935).
Vesicular stomatitis	85	Galloway and Elford (1933).
Fowl plague	75	Elford and Todd (1933).
Bacteriophages, *Staphylococcus* K, D4, D12	60	Elford and Andrewes (1932).
Bacteriophages, D54, and S41	35	Elford and Andrewes (1932).
Bacteriophage, *B. megatherium*	35	Tang, Elford, and Galloway (1937).
Rift Valley fever	30	Broom and Findlay (1933).
Tobacco mosaic disease	30	Smith (1936).
Rabbit papilloma of Shope	29	Schlesinger and Andrewes (1937).
Equine encephalomyelitis	28	Tang, Elford, and Galloway (1937).
Bacteriophage, *B. typhosus* T111	25	Yaoi and Sato (1935).
St. Louis encephalitis	25	Elford and Perdrau (1935).
Haemocyanin (Helix)	23	Elford and Ferry (1936).
Yellow fever (neurotropic and viscerotropic strains)	23	Findlay and Broom (1933).
Louping-ill	17	Elford and Galloway (1933*a*).
Bacteriophage, S13	10	Elford and Andrewes (1932).
Foot-and-mouth disease	10	Galloway and Elford (1931).
Poliomyelitis	10	Elford, Galloway, and Perdrau (1935).
Edestin	8	Elford and Ferry (1936).
Serum pseudo-globulin	7	Elford and Ferry (1934).
Serum albumin	5	Elford and Ferry (1934).
Oxyhaemoglobin	4·5	Elford (1933).
Egg albumin	4	Elford (1933).

REFERENCES

AMIES, C. R. (1934), *Brit. J. exp. Path.*, **15**, 314.

ARAGÃO, H. DE B. (1911), *Brazil-med.*, **25**, 471.

—— (1911*a*), *Mem. Inst. Osw. Cruz.*, **3**, 309.

BARNARD, J. E., and ELFORD, W. J. (1931), *Proc. roy. Soc.*, B, **109**, 360.

BAUER, J. H., and COX, H. R. (1935), *Proc. Soc. exp. Biol., N.Y.*, **32**, 567.

BAUER, J. H., COX, H. R., and OLITSKY, P. K. (1935), *Proc. Soc. exp. Biol., N.Y.*, **33**, 378.

BAUER, J. H., FITE, G. L., and WEBSTER, L. T. (1934), *Proc. Soc. exp. Biol., N.Y.*, **31**, 696.

BAUER, J. H., and HUGHES, T. P. (1934), *J. gen. Physiol.*, **18**, 143.

BORREL, A. (1904), *C.R. Soc. Biol., Paris*, **57**, 642.

BROOM, J. C., and FINDLAY, G. M. (1933), *Brit J. exp. Path.*, **14**, 179.

—— —— (1936), *Brit. J. exp. Path.*, **17**, 135.

BURNET, F. M., and BARNARD, J. E. (1933), *J. Path. Bact.*, **37**, 107.

BURNET, F. M., and FERRY, J. D. (1934), *Brit. J. exp. Path.*, **15**, 56.

COLES, A. C. (1929), *Brit. med. J.*, **2**, 91.

—— (1930), *Lancet*, **1**, 1011.

ELFORD, W. J. (1933), *Proc. roy. Soc.*, B, **112**, 384.

—— (1936), *Brit. J. exp. Path.*, **17**, 399.

ELFORD, W. J., and ANDREWES, C. H. (1932), *Brit. J. exp. Path.*, **13**, 446.
—— —— (1932 *a*), *Brit. J. exp. Path.*, **13**, 36.
—— —— (1935), *Brit. J. exp. Path.*, **16**, 61.
—— —— (1936), *Brit. J. exp. Path.*, **17**, 422.
ELFORD, W. J., ANDREWES, C. H., and TANG, F. (1936), *Brit. J. exp. Path.*, **17**, 51.
ELFORD, W. J., and FERRY, J. D. (1934), *Bio-chem. J.*, **28**, 650.
—— —— (1936), *Bio-chem. J.*, **30**, 84.
ELFORD, W. J., and GALLOWAY, I. A. (1933), *Brit. J. exp. Path.*, **14**, 196.
—— —— (1933 *a*), *J. Path. Bact.*, **37**, 381.
—— —— (1936), *J. Hyg., Camb.*, **36**, 536.
—— —— (1937), *Brit. J. exp. Path.*, **18**, 155.
ELFORD, W. J., GALLOWAY, I. A., and PERDRAU, J. R. (1935), *J. Path. Bact.*, **40**, 135.
ELFORD, W. J., and PERDRAU, J. R. (1935), *J. Path. Bact.*, **40**, 143.
ELFORD, W. J., PERDRAU, J. R., and SMITH, W. (1933), *J. Path. Bact.*, **36**, 49.
ELFORD, W. J., and TODD, C. (1933), *Brit. J. exp. Path.*, **14**, 240.
FINDLAY, G. M., and BROOM, J. C. (1933), *Brit. J. exp. Path.*, **14**, 391.
FINDLAY, G. M., MACKENZIE, R. D., and MACCALLUM, F. O. (1938), *Trans. R. Soc. trop. Med. Hyg.*, **32**, 183.
GALLOWAY, I. A., and ELFORD, W. J. (1931), *Brit. J. exp. Path.*, **12**, 407.
—— —— (1933), *Brit. J. exp. Path.*, **14**, 400.
—— —— (1936), *J. Hyg., Camb.*, **36**, 532.
GORDON, M. H. (1937), *Edinb. med. J.*, **44**, 65.
HALBERSTAEDTER, L. (1912), *Prowazek's Handbuch der pathogenen Protozoen*, p. 172, Leipzig.
HALBERSTAEDTER, L., and VON PROWAZEK, S. (1907), *Dtsch. med. Wschr.*, **2**, 1285.
HERZBERG, K. (1936), *Klin. Wschr.*, **2**, 1385, 1665.
JULIANELLE, L. A., HARRISON, R. W., and LANGE, A. C. (1938), *Amer. J. Path.*, **14**, 579.
KLING, C., PAÏC, M., KRASSNOFF, D., and HABER, P. (1938), *Ann. Inst. Pasteur*, **60**, 259.
KRASSNOFF, D., and REINIÉ, L. (1937), *C.R. Soc. Biol., Paris*, **124**, 790.
LEDINGHAM, J. C. G. (1931), *Lancet*, **2**, 525.
LEVADITI, C., PAÏC, M., and KRASSNOFF, D. (1936 *a*), *C.R. Soc. Biol., Paris*, **122**, 526.
—— —— —— (1936 *b*), *C.R. Soc. Biol., Paris*, **123**, 1048.
LEVADITI, C., PAÏC, M., KRASSNOFF, D., and VOET, J. (1936), *C.R. Soc. Biol., Paris*, **122**, 619.
LEVINTHAL, W. (1930), *1st Congr. int. Microbiol.*, Masson, Paris.
—— (1935), *Lancet*, **1**, 1207.
LILLIE, R. D. (1930), *Publ. Hlth. Rep., Wash.*, **45**, 773.
LINDNER, K. (1909), *Wien. klin. Wschr.*, **2**, 1555.
—— (1910), *Wien. klin. Wschr.*, **1**, 283.
—— (1911), *v. Graefes Arch. Ophthal.*, **78**, 345.
LIPSCHÜTZ, B. (1911), *Arch. Derm. Syph., Wien.*, **107**, 387.
MCINTOSH, J., and SELBIE, F. R. (1937), *Brit. J. exp. Path.*, **18**, 162.
MACKIE, T. J., and VAN ROOYEN, C. E. (1937), *Edinb. med. J.*, **44**, 72.
MARCHAL, J. (1930), *J. Path. Bact.*, **33**, 713.
MERLING-EISENBERG, K. B. (1938), *Brit. J. exp. Path.*, **19**, 338.
MIYAGAWA, Y., MITAMURA, T., YAOI, H., ISHNI, N., and OKANISHI, J. (1935), *Jap. J. exp. Med.*, **13**, 723.
MIYAGAWA, Y., *et al.* (1935 *a*), *Jap. J. exp. Med.*, **13**, 1, 331, 723, 733.
NAUCK, E. G., and PASCHEN, E. (1933), *Zbl. Bakt.*, Abt. I, orig., **128**, 171.
PAÏC, M., KRASSNOFF, D., and REINIÉ, L. (1938), *Ann. Inst. Pasteur*, **60**, 250.
PAÏC, M., KRASSNOFF, D., HABER, P., REINIÉ, L., and VOET, J., (1938), *Ann. Inst. Pasteur*, **60**, 227.
PASCHEN, E. (1906), *Münch. med. Wschr.*, **53**, 2391.
—— (1913), *Dtsch. med. Wschr.*, **39**, 2135.
—— (1917), *Dtsch. med. Wschr.*, **43**, 746.
—— (1933), *Zbl. Bakt.*, Abt. I, orig., **130**, 190.
—— (1936), *Zbl. Bakt.*, Abt. I, orig., **135**, 445.
—— (1937), *Zbl. Bakt.*, Abt. I, orig., **138**, 1.
RIVERS, T. M., and SCOTT, T. F. MCN. (1936), *J. exp. Med.*, **63**, 415.
SCHLESINGER, M., and ANDREWES, C. H. (1937), *J. Hyg., Camb.*, **37**, 521.
SHORTT, H. E., PANDIT, C. G., and RAO, R. S. (1938), *Indian J. med. Res.*, **26**, 229.
SMITH, K. M. (1936), *Science Progress*, No. 119 (January).
TANG, F. F., ELFORD, W. J., and GALLOWAY, I. A. (1937), *Brit. J. exp. Path.*, **18**, 269.
TANIGUCHI, T., HOSOKAWA, M., KUGA, S., and MASUDA, Z. (1934), *Jap. J. exp. Med.*, **12**, 101.
TANIGUCHI, T., *et al.* (1935), *Jap. J. exp. Med.*, **13**, 109.
—— (1935 *a*), *Jap. J. exp. Med.*, **13**, 577.

PART II.

INCLUSION BODIES.

A special study has been made of the inclusions of vaccinia and of molluscum contagiosum. Observations on vaccinial keratitis in rabbits has revealed that in addition to intracytoplasmic inclusion bodies in corneal epithelium, identical inclusion bodies were found to occur within fibroblast cells of the substantia propria. The experiments were carried a step further and observations were confirmed by obtaining a tissue culture of fibroblast cells, infecting it with vaccinia virus, and thereafter watching the formation of acidophile inclusions within these cells. The possibility of the appearance described being artefacts, was thus excluded. The latter research is described in Part IV of this thesis.

Before micromanipulation studies were possible it was first necessary to design an instrument suitable for the purpose, and thereafter to acquire sufficient proficiency in its operation. About one year was occupied in perfecting methods, following which, microdissections were carried out on the inclusion bodies of vaccinia, ectromelia, psittacosis, fowl-pox and molluscum contagiosum.

Investigations on the first three are incomplete, but the micromanipulation and dissection of the molluscum body was successfully accomplished and the findings/

findings duly published. The chemical composition of the latter was also examined, and the information obtained was of interest in so far that similar findings had been made regarding the composition of the inclusions of trachoma and inclusion conjunctivitis.

See Part

I would like to mention that owing to the extreme difficulty of this class of investigation, progress is painfully slow, and frequently much labour yielded but a small reward. A complete study of all inclusions by microdissection would occupy the greater part of a life time. Infected tissues containing inclusion bodies large enough and numerous enough for dissection are often difficult to secure. For instance, when working with Rabies virus 10 different strains of rabies virus (obtained from Professors Remlinger and Levaditi in Paris) were inoculated into rabbits and films from the hippocampus major were searched for Negri bodies but, despite many efforts, the viruses tested proved to be ~~only~~ feebly Negrigenic and microdissection had to be abandoned. The use of sheep or monkeys might have provided one with larger Negri bodies, but the handling of these animals is not without personal risk of infection.

THEILER, M., and BAUER, J. H. (1934), *J. exp. Med.*, **60**, 767.
THYGESON, P. (1934), *Arch. Ophthal., N.Y.*, **12**, 307.
—— (1934*a*), *Amer. J. Ophthal.*, **17**, 1019.
THYGESON, P., and MENGERT, W. F. (1936), *Arch. Ophthal., N.Y.*, **15**, 377.
THYGESON, P., and PROCTOR, F. T. (1935), *Arch. Ophthal., N.Y.*, **13**, 1018.
TILDEN, E. B., and GIFFORD, S. R. (1936), *Arch. Ophthal., N.Y.*, **16**, 51.
VAN ROOYEN, C. E. (1937), *Zbl. Bakt.*, Abt. I, orig., **139**, 130.
VON PROWAZEK, S. (1911), *Arch. Schiffs- u. Tropenhyg.*, **15**, 173.
WOODRUFF, C. E., and GOODPASTURE, E. W. (1929), *Amer. J. Path.*, **5**, 1.
—— —— (1930), *Amer. J. Path.*, **6**, 713.
YAOI, H., KANAZAWA, K., and SATO, K. (1936), *Jap. J. exp. Med.*, **14**, 73.
YAOI, H., and NAKAHARA, W. (1935), *Jap. J. exp. Med.*, **13**, 757.
YAOI, H., and SATO, K. (1935), *Jap. J. exp. Med.*, **13**, 565.

CHAPTER VII

INCLUSION BODIES

THE majority of virus diseases are characterized by the occurrence of organized structures in the cytoplasm or the nucleus of infected tissue cells. These structures are known as inclusion bodies, inclusions, or virus inclusions; it is necessary to define what is meant by these terms. Histologists sometimes use the term 'inclusion body' in a broad sense, to refer to any structure in the cytoplasm of a cell other than such purely normal constituents as the mitochondria, Golgi apparatus, or centrosome; thus, they might refer to globules of fat or particles of glycogen as inclusion bodies. Virus workers use the term in a restricted sense, to refer to peculiar intracellular structures generally regarded as characteristic of virus infections. These inclusions may occur in the nucleus or cytoplasm, and be eosinophilic or basophilic. The question of terminology is somewhat complicated, however, by the fact that bodies similar to those occurring in virus infections may occur intracellularly in tissues which are either healthy, or else affected by a disease of non-virus aetiology. For example, while one talks about the Negri body as the characteristic inclusion body of rabies, one also says that inclusion bodies have been found in whooping-cough and brain tumours, both probably non-virus conditions. We find the term 'virus inclusion' a useful one, but its use does not imply that we necessarily believe all such structures to be composed of virus particles.

In this chapter we shall discuss the various types of inclusion, the occurrence of similar bodies in non-virus diseases, the experimental production of inclusions, and the methods used to elucidate their precise nature. We shall also discuss the biological nature of inclusions. This chapter is only intended to serve as an introduction to the subject, for each individual inclusion is usually treated at length in its appropriate chapter. In this chapter we intend to discuss the inclusions of human viruses, and the better-known inclusions of animal viruses; inclusions have also been reported in various diseases of plants, fish, and insects (see Rivers, 1928; Ludford, 1930; Smith, 1930; St. John-Brooks, 1930), but these do not properly fall within our scope and no mention of them will be made.

Concerning literature, a number of valuable papers of a general nature have appeared in recent years, some giving illustrations (Findlay and Ludford, 1926; Rivers, 1928; Ludford, 1930; Cowdry, 1934; Ishimitsu, 1937).

Classification. Various types of classification may be used in describing these structures. For example, Lipschütz (1921) divided inclusions into three groups: the cyto-oikon or intracytoplasmic body; the karyo-oikon or intranuclear body; and the cyto-karyo-oikon, the body that occurs in both sites. This terminology, however, is scarcely used at the present: it is so much easier to refer simply to intracytoplasmic or intranuclear inclusions.

Cowdry (1934) has published a valuable classification of intranuclear bodies, which are divided into Types A and B, according to morphology.

THE MORPHOLOGY OF INCLUSION BODIES

Intracytoplasmic

These bodies occur in various forms. They may be large eosinophilic structures; they may be small basophilic granules; or they may belong

to the group of inclusions resembling the trachoma body. Less common types are small eosinophilic or large basophilic structures.

(*a*) *Large eosinophilic inclusions* measure from about 5 μ up to 20 μ in diameter. They are usually spherical, but may be oval, elongated, or triangular. These bodies commonly appear more or less homogeneous, but special methods (*vide infra*) may detect granularity. The better-known virus inclusions of this type are the following: the Guarnieri body of variola-vaccinia (see p. 318; Plate III B); the Negri body of rabies (see Chapter LII; Plate III A); the Henderson-Paterson body of molluscum contagiosum (see p. 123; Plate I); the Bollinger body of fowl-pox; and the Marchal body of mouse ectromelia.

(*b*) *Small eosinophilic inclusions.*. These inclusions are usually early forms of the above. Sometimes the granules become clumped together to form a larger structure. Such small granules may be found in rabies and vaccinia, and have been reported in mumps (see p. 245).

(*c*) *Small basophilic inclusions.* These inclusions are usually formed by a collection, as it were a 'colony', of countless small elementary bodies. The typical appearance is usually of discrete blue granules lying in a more or less circumscribed area of the cytoplasm. Small basophilic inclusions occur in encephalitis lethargica (see p. 837); herpes febrilis (see p. 157); lymphogranuloma inguinale (see p. 186; Plate II *i* and *j*).

(*d*) *Large basophilic inclusions.* Large basophilic homogeneous inclusions have been described in lymphogranuloma inguinale (Gamna-Favre bodies). Studies have shown, however, that they are probably nuclear in origin, and not true virus inclusions (see p. 181). This type of inclusion is only rarely found, and is not characteristic of virus infections.

(*e*) *The trachoma type of inclusion.* The inclusion bodies of trachoma, inclusion conjunctivitis, and psittacosis have so many features in common that they can conveniently be described together (see Plates IV and II *a–h*). Thus, with certain minor differences, they each undergo a series of distinctive changes whilst in process of development; they stain readily with Giemsa's solution; and during the early stages of growth they appear as basophilic intracytoplasmic structures. As time proceeds, the inclusions not only increase in size and alter in shape, but also exhibit a tendency to acidophilic characters. The mature inclusion body consists of a clump of pink-stained elementary bodies which are sometimes covered by a film of cell material stained light blue, so that the structure as a whole has a lilac tint. This appearance is most marked in the inclusion bodies of trachoma and those of inclusion blennorrhoea, and is less obvious in the case of psittacosis virus.

During the last decade the inclusions of psittacosis have been exhaustively investigated by Bedson and Bland (see p. 585), who showed that the earliest visible growth phase of the parasite took the form of a large, apparently homogeneous, basophilic and intracytoplasmic structure which they called the plaque or plasmodium stage. As growth progressed the plaque grew in size and underwent segmentation, so that it eventually assumed the appearance of a segmented mass, called a morula. Owing to the resemblance of these initial growth phases of the virus to the early multiplication forms of a protozoal bird parasite, Bedson (see p. 586) at first expressed the view that the psittacosis virus showed morphological similarities more closely related to the protozoa than to the bacteria. Subsequent work by Bedson and Bland (see p. 587) dispelled this theory, for they showed that, although the earliest developmental forms of the virus seemed to be homogeneous in structure, they were in reality composed of an aggregation of smaller masses. As the

inclusions matured, minute pink-staining elementary bodies began to develop within them and eventually these filled the whole cell, which subsequently ruptured and discharged its contents into the surrounding tissue spaces. The free-lying virus bodies next entered healthy endothelial cells and commenced a second life cycle.

The inclusion bodies of psittacosis, trachoma, and inclusion conjunctivitis constitute a unique group of structures, and owing to the facility with which they can be demonstrated in tissues and the colour effects which are obtainable in film preparations, they have been examined by cytologists, certain of whom have suggested that the appearances of the inclusion body at different stages of its growth are not solely produced by the parasite itself but are also contributed to by the cell. Levinthal (see p. 587), for instance, goes so far as to assert that the initial forms referred to as the plaque, plasmodium, and morula varieties represent, to a large extent, the reaction of the parasitized cell and, to a lesser degree, the virus itself. This opinion is somewhat similar to that expressed in the past by early German writers in respect of the nature of the trachoma inclusion body. They, for example, pointed out that the light blue-staining masses which developed in the cytoplasm of invaded epithelial cells after infection represented a product of cell degeneration, on account of which they called them 'plastin masses'. The successive developmental stages of the trachoma inclusion body have been investigated in great detail by Halberstaedter and Prowazek (see p. 610), and from their survey of the literature on this subject they concluded that the first sign of growth of the virus was the formation of the initial body, inside which in turn elementary bodies, or *Strongyloplasma* as they were called at the time, began to appear. This process started in the initial bodies situated at the centre of the cytoplasm and gradually involved those situated peripherally, so that eventually the whole cytoplasm became filled to bursting-point with elementary bodies, the nucleus became pushed to one side, and the cell ruptured.

Much of what has been said regarding the morphology of the Halberstaedter-Prowazek inclusion body is equally applicable to the inclusion body of inclusion blennorrhoea, and for all practical purposes they may be regarded as identical in appearance. The only outstanding difference between the two inclusions lies in their relative distribution in affected tissues, for whereas in trachoma the bodies only occur in the conjunctival epithelium, the virus of blennorrhoea has also been demonstrated in the mucous membrane of the cervix uteri and, on rare occasions, in the male urethra.

An interesting observation which has been made regarding this group of inclusions is the fact that the elementary bodies appearing in the fully developed trachoma inclusion body are embedded in a jelly-like material: this substance sometimes clings to the elementary bodies after they have been liberated. It is this mucoid covering which produces the halo-like zone of non-staining material that surrounds individual elementary bodies in Giemsa-stained films (see p. 614).

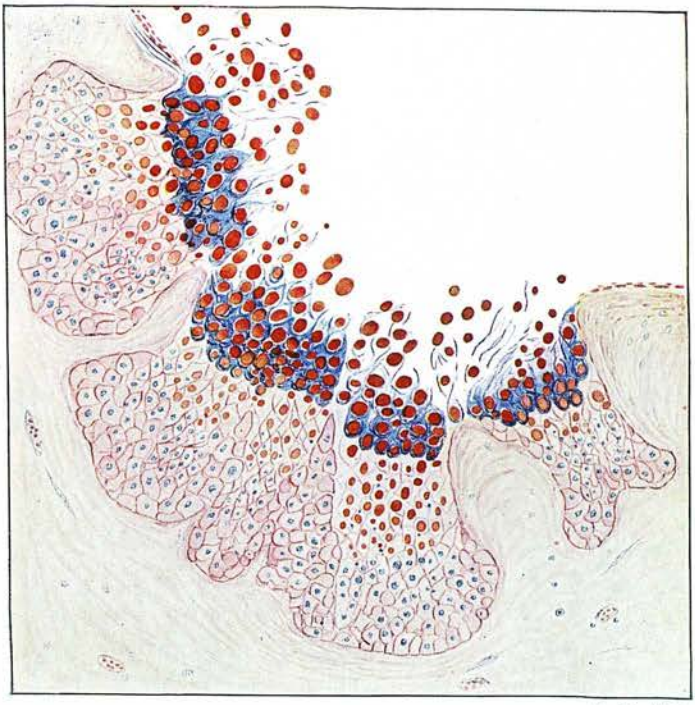
The researches of Bland and Canti (see p. 588), who obtained a cinephotomicrographic record of the growth phases of psittacosis virus in tissue cultures, also revealed that the inclusion body possessed a definite outer covering or envelope composed of a relatively dense substance (see p. 589).

The microchemical reactions of the inclusion body of trachoma have been investigated by Rice (see p. 614), who found that the matrix of the structure consisted of a carbohydrate substance which yielded a specific

PLATE I

Histological section through a skin nodule derived from a case of molluscum contagiosum. Observe the numerous molluscum (or Henderson-Paterson) inclusion bodies, which are coloured dark red. Stained by Mann's method. × 100 (approx.).

PLATE I



C.E.v.R. and A.Z.R.

colour reaction when treated with Lugol's iodine solution; furthermore, the inclusion body could be deprived of this property by preliminary exposure to the digestive action of saliva. The inclusion body of inclusion conjunctivitis has also been found to react in a similar manner.

At the time of writing, interest has been focused on the inclusion and elementary bodies of lymphogranuloma inguinale, in view of a claim put forward by Findlay and his co-workers (1938) that this virus also undergoes a developmental process similar to that of psittacosis. A full description of work on the subject appears on p. 186, but although certain developmental phases of the inclusions of lymphogranuloma inguinale unquestionably resemble those of psittacosis virus, we would suggest that further work is required before definite conclusions can be reached.

Intranuclear

Cowdry (1934) states that the characteristic features of these inclusions are as follows: eosinophilic staining reaction; presence of an unstained halo between the inclusion and the nuclear membrane; and margination of basichromatin on the nuclear membrane. Cowdry also reports that intranuclear inclusions can be divided, on the basis of morphology, into Types A and B.

Type A inclusions are either amorphous, granular, or occur in rounded masses. The nuclear material is severely disorganized, and the basichromatin collects on the nuclear membrane. These inclusions contain no iron or thymonucleic acid, and micro-incineration leaves no ash. Affected tissues show bodies in all stages of development; thus, certain cells may contain fully developed inclusions, while nearby only very small forms may occur. There is usually a severe tissue reaction in the neighbourhood of Type A bodies. Type A inclusions are found most characteristically in herpes febrilis; yellow fever (see p. 469); chickenpox (see p. 255); zoster (see p. 140); and virus III infection of rabbits.

Type B inclusions cause much less reaction in the nucleus than do Type A. The inclusions themselves are more circumscribed; they vary in size and there may be a number in any one nucleus; there is no margination of basichromatin; and there is no marked tissue reaction in the surrounding areas. Type B inclusions occur, for example, in poliomyelitis (see p. 795); Rift Valley fever (see p. 415); and Borna disease of horses.

VIRUS DISEASES OF MAN IN WHICH INCLUSION BODIES OCCUR

Intracytoplasmic

Eosinophilic. Encephalitis lethargica (Dawson's bodies, see p. 838); Japanese encephalitis (Taniguchi's bodies, see p. 868); molluscum contagiosum (Henderson-Paterson bodies, see p. 123; Plate I); mumps (see p. 245); rabies (Negri bodies, see Ch. LII; Plate III); variola-vaccinia (Guarnieri bodies, see p. 318; Plate III); warts (see p. 134).

Basophilic. Encephalitis lethargica (Da Fano's bodies, see p. 837); herpes febrilis (Da Fano's and Nicolau's bodies, see p. 157); inclusion conjunctivitis (see p. 632; Plate IV); lymphogranuloma inguinale (Gamna-Favre bodies, Miyagawa's bodies, see p. 186; Plate II); measles (see p. 220); psittacosis (see p. 585; Plate II); trachoma (see p. 610; Plate IV).

Intranuclear

Type A. Herpes febrilis (Lipschütz bodies, see p. 160); herpes zoster (Lipschütz bodies, see p. 140); varicella (see p. 255); virus 'B' infection (see p. 890); yellow fever (see p. 469).

Type B. Herpes febrilis (Nicolau's bodies, see p. 160); herpes zoster (Nicolau's bodies, see p. 140); poliomyelitis (see p. 760); Rift Valley fever (see p. 415).

CELL INCLUSIONS FOUND IN HUMAN TISSUES IN NON-VIRUS DISEASES

Inclusions have been described in certain conditions in which no virus is held to occur. Thus intranuclear inclusions have been found in nerve cells of persons dying from different diseases (Wolf and Orton, 1932). Nuclear inclusions were found in the tubular epithelium of the kidney and in liver cells in a number of cases of lead poisoning in children (Blackman, 1936). A number of authors have described Type A intranuclear inclusions in the lungs of children dying from whooping-cough pneumonia (Rich, 1932; Kuttner and Wang, 1934; McCordock and Smith, 1934). Goodpasture *et al.* (1939) described inclusions in the nuclei of epithelial cells in the respiratory tract of infants after measles or whooping-cough. Broadhurst *et al.* (1939) found inclusions in the respiratory system and blood cells after scarlet fever.

Gliomata, mainly those of the spongioblastoma multiforme type, were found to contain large eosinophilic intracytoplasmic inclusions in 33 per cent. of cases (Russell, 1932).

INTRACELLULAR INCLUSIONS FOUND IN HEALTHY HUMAN TISSUES

Structures resembling virus inclusions have been found in the tissues of healthy persons on a number of occasions.

1. Type A intranuclear inclusions were found in the intestines, liver, and lungs of a man aged 36 (VonGlahn and Pappenheimer, 1925).

2. Type B inclusions have been found in the kidneys in a number of healthy human beings (Cowdry *et al.*, 1935).

3. A number of authors have described intranuclear inclusions in healthy young children (e.g. Goodpasture and Talbot, 1921; Farber and Wolbach, 1932; Kuttner and Wang, 1934; Pappenheimer and Hawthorne, 1936; for other references see VonGlahn and Pappenheimer, 1925). The patients were usually infants; sometimes still-born foetuses were examined. The inclusions occurred in the bile-ducts, intestine, kidneys, liver, lungs, pancreas, parotids, thyroid, and respiratory tract. Farber and Wolbach found both nuclear and cytoplasmic inclusions in 12 per cent. of submaxillary glands examined in infancy. Similar intranuclear bodies were found by Kuttner and Wang (1934).

CELL INCLUSIONS OBSERVED IN ANIMAL VIRUS DISEASES

Intracytoplasmic

Eosinophilic. Canary-pox; dog distemper; ectromelia (Marchal bodies); foot-and-mouth disease in animals; fowl-pox (Bollinger bodies); louping-ill (see p. 879); myxomatosis of rabbits; ferret epizootics (Slanetz and Smetana, 1937; Spooner, 1938); fowl plague; sheep-pox; cow-pox.

Intranuclear[1]

Type A. Fox encephalitis; louping-ill (see Ch. LXIV); infectious tracheitis of chickens; an owl disease; parrot and parakeet disease; pseudo-

[1] Many of these inclusions have been personally studied by Cowdry (1934).

PLATE II

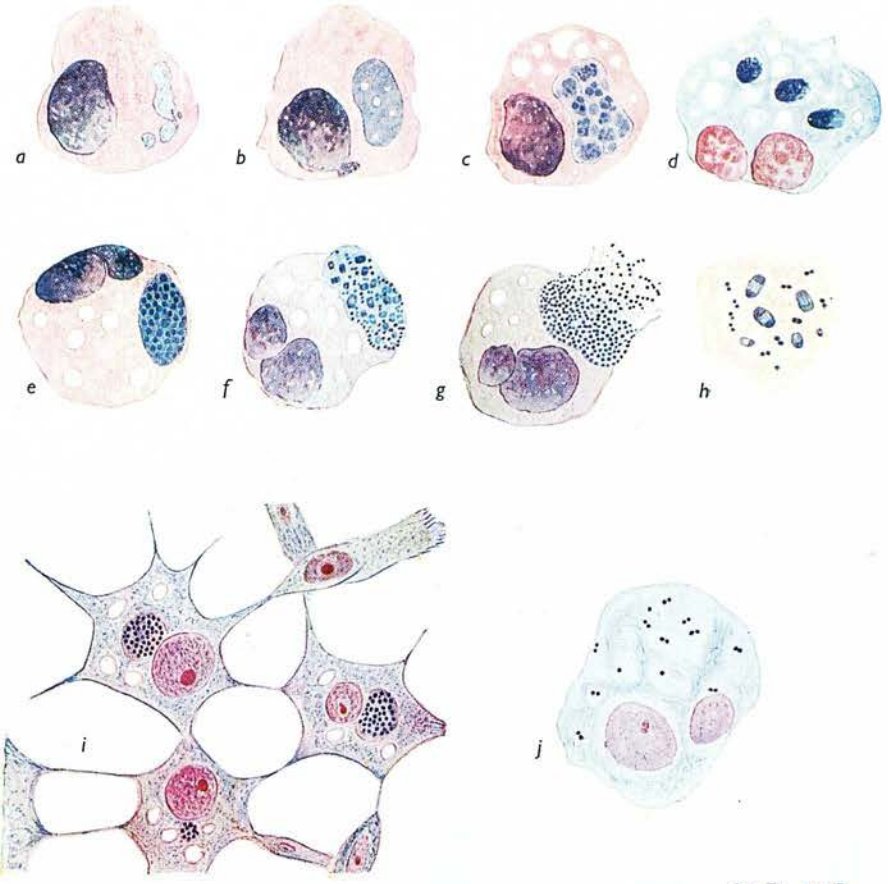
a, *b*, *c*, *d*, *e*, *f*, *g*, and *h*, illustrate different morphological growth phases of psittacosis virus. Each variety is described individually in Chapter XLV.

i. Tissue culture of rabbit corneal epithelium infected with lymphogranuloma inguinale virus. Observe the two large and one small clumps of elementary bodies. These have been referred to in the literature as Miyagawa's granulo-corpuscles.

j. Histiocyte from meninges of mouse infected with lymphogranuloma inguinale virus, showing Miyagawa's granulo-corpuscles, some of which occur singly and others in pairs.

All the above have been drawn from microscopic preparations stained with Giemsa's solution, and are magnified about × 1,200.

PLATE II



C.E.J.R. and A.J.R.

rabies (see p. 744); rats infected with an active principle in sewage (Hindle and Stevenson, 1929–30; Hindle, 1932); salivary gland virus of guinea-pigs; salivary gland virus of mice; salivary gland virus of rats; virus III of rabbits.

Type B. Borna disease of horses; infection of the livers of mice (Findlay, 1932).

CELL INCLUSIONS OCCURRING IN HEALTHY ANIMAL TISSUES

Inclusions have been found in a number of apparently healthy animals; it should be realized, however, that viruses may be present in these conditions as it is now known that there exist a number of more or less benign virus infections of animals.

Monkeys. Intranuclear inclusions have been found in the respiratory system and bile-ducts (Covell, 1932), in the kidneys (Cowdry and Scott, 1935 *b*), and in the liver cells (Pappenheimer and Hawthorne, 1936).

Dogs. Type A intranuclear inclusions were demonstrated in the liver cells of two dogs by Cowdry and Scott (1930). Nuclear inclusions were demonstrated in the liver and kidney cells of dogs over two years of age (Nicolau and Kopciowska, 1936).

Cats. Normal cats may show small intracytoplasmic granules in their nerve cells (Negri-Luzzani, 1905, 1913).

Guinea-pigs. Pappenheimer and Hawthorne (1936) found nuclear inclusions in the liver cells.

Mice. Negri-like bodies may occur in the nerve cells (Nicolau, Kopciowska, and Balmus, 1933).

Ferrets. Inclusion bodies were found in the nuclei of the liver cells by Pappenheimer and Hawthorne (1936).

Moles. Intranuclear bodies were found in the salivary glands by Rector and Rector (1934).

Birds. Type A lesions were found in the kidneys of a Guatemalan amazon; Type B lesions were found in the lungs of this species and the kidneys of a number of other birds (Cowdry *et al.*, 1935).

Frogs. Cowdry (1934) found Type A lesions in the kidneys of frogs.

THE PRODUCTION OF INCLUSIONS BY PHYSICAL AND CHEMICAL AGENTS

A number of physical and chemical factors have been said to cause inclusions when inoculated into experimental animals.

Intracytoplasmic. 1. Russell's viper venom produced Negri-like bodies in nerve cells in guinea-pigs (Acton and Harvey, 1911).

2. *B. pyocyaneus* injections produced Negri-like bodies in rabbits (Acton and Harvey, 1911).

3. Prolonged administration of pilocarpine produced Negri-like bodies in the salivary glands (Shortt and Lahiri, 1934).

Intranuclear. 1. Inclusions were produced in guinea-pigs by injections of certain aluminium, carbon, and iron compounds; these structures gave negative Feulgen and MacCallum reactions, and so were clearly not of nuclear origin (Olitsky and Harford, 1937).

2. Bismuth injections gave rise to nuclear inclusions in rats (Pappenheimer and Maechling, 1934).

3. Lee (1933–4) produced intranuclear inclusions in the nerve cells of cats after intravenous injection of glucose, common salt, sodium bicarbonate, and distilled water. After injection of salyrgan, he found inclusions in the pancreas, kidneys, testes, and suprarenals. In a more detailed

study (1936) he produced Type B lesions in the cells of the anterior horn by intravenous injection of distilled water. Following injection of hypertonic glucose, glucose plus acacia, common salt, and sodium bicarbonate, Type A lesions developed in Purkinje cells, in anterior horn cells, in cells of the spinal and sympathetic ganglia, and in pyramidal cells.

4. Nuclear inclusions were produced in the kidneys of guinea-pigs, mice, and rats by the administration of lead; these structures gave a negative Feulgen reaction (Blackman, 1936).

5. Intranuclear inclusions developed in nerve cells after prolonged electrical stimulation of nerves in turtles, cats, and monkeys (Heinbecker and O'Leary, 1930).

6. Soaking nerve tissue in hypertonic saline produced nuclear inclusions (Davenport *et al.*, 1931).

7. Administration of irradiated ergosterol produced intranuclear inclusions in the kidneys of rhesus monkeys, and in the parotid and submaxillary glands of cebus monkeys (Cowdry and Scott, 1935 *a*).

METHODS USED IN THE STUDY OF INCLUSIONS

1. *Microscopic examination.* Inclusion bodies may be examined in wet preparations by the dark-ground microscope, with a view to detecting granularity; infra-red microscopy has also been employed. Both these methods have shown, for example, that the Negri body of rabies is a granular structure, and not homogeneous, as it appears in stained films (see Ch. LII). Recently Himmelweit (1938) has employed a very delicate microscopical method (see p. 13); he studied *in situ* by the Heine Ultropak (Leitz) microscope the chorio-allantois of ducks' eggs infected with vaccinia (and ectromelia). He found that in infected epithelial cells there were large numbers of granules, which increased in number, and were always contained in a matrix. There is little doubt that the granules were virus elementary bodies, and that the whole accumulation represented a Guarnieri body.

As a general rule, however, inclusions are examined microscopically only after staining. Staining methods are applied for two main reasons.[1] First, as a means of demonstrating accurately the morphology of the body in question. Secondly, stains and other reagents are applied to test, as far as possible, the composition of the inclusion; these tests are as follows:

(*a*) The Feulgen technique is performed as a test for thymonucleic acid; any structure giving a positive reaction contains chromatin. True virus inclusions should give a negative Feulgen reaction, whether they occur in the cytoplasm or the nucleus.[2] Ingested nuclei may sometimes resemble virus inclusions, but their true nature can be detected by this reaction; for example, the so-called Gamna-Favre bodies of lymphogranuloma inguinale give a positive reaction, showing that they are of nuclear origin.

(*b*) MacCallum's test detects the presence of masked iron, a positive reaction signifying the presence of nuclear chromatin.

(*c*) The application of hydrochloric acid usually dissolves nuclear chromatin, but leaves inclusions intact.

(*d*) Stains for mitochondria, the Golgi apparatus, and the centrosome prevent smaller forms of inclusion body from being confused with these normal intracellular structures.

(*e*) The oxidase reaction detects the presence of granules of leucocytes, which give a blue colour. Sometimes a flattened leucocyte may simulate a fibroblast with eosinophilic inclusions; sometimes also histiocytic cells

[1] For the technique of staining, a text-book of histology should be consulted.

[2] See, however, recent work on vaccinial inclusions (p. 323).

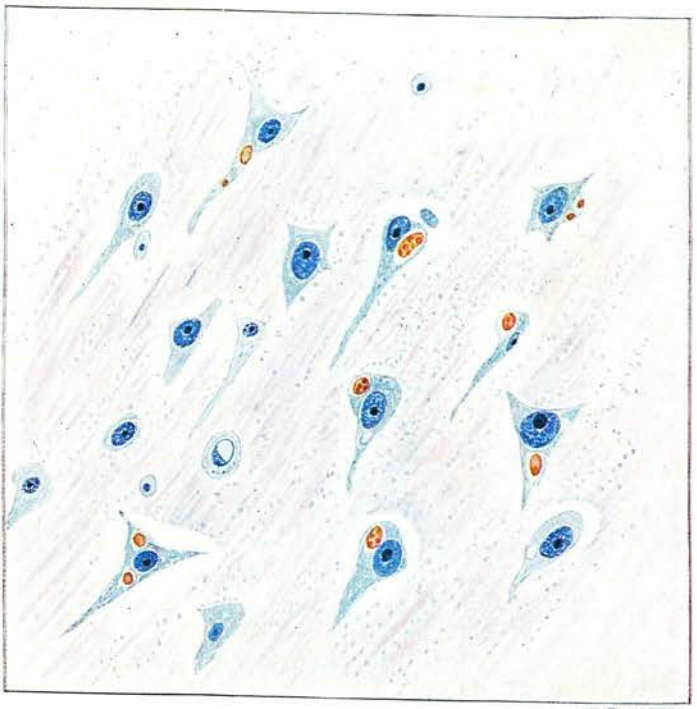
PLATE III

A. Histological section through the hippocampus major of a dog suffering from hydrophobia. Observe the red-stained Negri inclusion bodies lying within the cytoplasm of nerve cells. The illustration has been prepared from a section of tissue stained by Mann's method, and is magnified approximately × 1,200.

B. Shows an histological section through the cornea of a rabbit, four days after scarification with vaccinia virus. Observe the red-stained Guarnieri bodies present in the cytoplasm of corneal epithelial cells. They vary considerably in size, being either homogeneous or granular in composition, and are often surrounded by an unstained halo. Stained by Mann's method, magnified approximately × 1,200.

PLATE III

A



C.E.v.R. and A.T.R.

B

C.E.v.R. and A.T.R.

may ingest the granules of degenerated leucocytes. In both these cases the oxidase reaction shows that the intracellular structures are not true virus inclusions, but only leucocytic granules.

(*f*) Intravital staining is sometimes carried out. Cresyl blue solution is applied to wet preparations of tissue containing inclusions, which take up the stain selectively, to appear distinctly blue.

It may be said that true virus inclusions give negative Feulgen[1] and MacCallum tests; resist solution in hydrochloric acid; do not react to tests for mitochondria, the Golgi apparatus, and the centrosome; do not give a positive oxidase test; and frequently stain vitally with cresyl blue.

2. *Tissue culture and egg culture.* Viruses may be added to the cells of a tissue culture, and the development of characteristic inclusions studied in wet, or preferably stained, preparations; the slide technique of growing cells is probably the best suited to this purpose (see also Ch. IX). By such means the inclusions of herpes febrilis (see p. 160), lymphogranuloma inguinale (see p. 186), psittacosis (see p. 588), and vaccinia (see p. 323) have been studied.

Viruses may also be added to fertile eggs (see also Ch. X); after an appropriate period of growth, the chorio-allantois is removed, and histological preparations made. By this technique the inclusions of herpes (see p. 160), lymphogranuloma (see p. 187), psittacosis (see p. 590), and vaccinia (see p. 322) have been studied. Andrewes (1930) has shown that the addition of immune serum prevents the development of herpetic inclusions (Lipschütz bodies).

3. *Animal inoculation.* Inclusion bodies are, of course, frequently studied experimentally in the tissues of infected animals; a particular application of this method has been used in the study of life cycles. For example, in the study of psittacosis, mice were injected with virus in the peritoneal cavity; animals were killed at short intervals thereafter, and preparations made from the spleen (Bedson and Bland, 1932, see p. 592). Working with lymphogranuloma inguinale, Findlay *et al.* (1938) injected mice intracerebrally; in this case also the animals were killed at short intervals thereafter. In both these studies it was possible to identify a life cycle by examination of successive specimens.

4. *Micro-incineration.* Of recent years, it has been found that important information can be yielded by subjecting tissue containing virus inclusions to a very high temperature; after incineration, slides are examined microscopically for the nature of the residual ash. Using this method, Scott (1930) found that the intranuclear inclusions of salivary gland disease of guinea-pigs left little or no ash, whereas nucleoli were found to be rich in ash. A method of distinguishing between intranuclear inclusions and nucleoli is thus available. Similar appearances are presented, after incineration, by the intranuclear inclusions of yellow fever (Cowdry, 1933), herpes (Rector and Rector, 1933), and Rift Valley fever (Horning and Findlay, 1934).

Danks (1932), studying intracytoplasmic bodies by micro-incineration, found that the Bollinger body of fowl-pox contained calcium salts, and was composed of numerous smaller granules, or Borrel bodies. Covell and Danks (1932) studied the Negri body, which yielded a compact white ash composed mainly of calcium.

5. *Ultracentrifugation.* This method was used by Lucas and Herrmann (1935) in their study of the Lipschütz body of herpes febrilis; they used the ultracentrifuge of Beams, Weed, and Pickels (see p. 47). A rabbit's cornea was excised, and cut into small pieces, 30 to 48 hours after inoculation

[1] See, however, p. 323.

with virus. The tissue was centrifuged for 45 to 60 minutes at 65 to 70 lb. air-pressure, fixed, and histological preparations made. The herpetic inclusions were, apparently, heavier than chromatin, and passed to the pole opposite to that where the chromatin was collected.

6. *Micromanipulation.* There is one method, beyond all others, of deciding whether an inclusion is a purely degenerative structure derived from the host, or whether it is infective, and derived from the parasite; that method is to isolate an inclusion by micromanipulation and then, if possible, inject it into a susceptible animal, eventually reproducing the infection.

The standard work on these lines was carried out by Woodruff and Goodpasture (1929), who isolated a fowl-pox inclusion by micromanipulation; it was then washed in saline, and finally injected into an animal. The washed inclusion reproduced the disease, while the saline used to wash the body proved non-infective. They further showed that an inclusion contained numerous elementary bodies. Baumgartner (1935 *a*) confirmed these results, and also worked with the inclusions of ectromelia of mice. She was able to reproduce the infection with a single washed ectromelia inclusion. Baumgartner (1935 *b*) has also carried out important work on the intranuclear Lipschütz body of herpes. The isolated body proved infective for the cornea, whereas the saline in which it was washed proved negative.

van Rooyen (1938) has applied this technique in another direction; he has carried out microdissection of the inclusion of molluscum contagiosum, and has shown that the body can be removed from the epithelial cell, and leave a definite socket therein. Also, it appears that the inclusion has a weak cap-like extremity, and that it contains numerous elementary bodies embedded in a sticky matrix (see also p. 129).

The significance of the above results is undoubted; it has been shown that two very typical intracytoplasmic inclusions, and one typical intranuclear inclusion, are infective, and must, therefore, contain the viable virus particles. These results have greatly influenced modern views on the nature of inclusions, and many feel that it is only a matter of time before other well-known inclusions, e.g. those of vaccinia, rabies, and yellow fever, will be treated in the same way and found to be infective.

THE SPECIFICITY OF VIRUS INCLUSIONS

We have described the appearance of a large number of inclusions occurring in virus diseases of man and animals. It has also been stated that apparently similar inclusions may be found in non-virus diseases of man, in healthy human beings and animals, and may be produced experimentally by various physical and chemical agents. If, then, inclusions occur in other conditions, to what extent do they indicate the presence of a virus? It should be noted that the morphological presence of an inclusion is only one test for the presence of a virus; this test should be combined with attempts to demonstrate the infectivity of the tissue in which the inclusions occur.

With regard to intracytoplasmic inclusions, we take up the position that a number are absolutely specific structures that do indicate the presence of a virus. They are so characteristic in their morphology, that they cannot be confused with other structures. Further, they are associated with the infectivity of the tissue in which they occur, and many develop in infected tissue cultures and eggs. Such inclusions are those of rabies, variola-vaccinia, molluscum contagiosum, lymphogranuloma inguinale (Miya-

PLATE IV

The drawings *a*, *b*, *c*, *d*, and *e*, show various growth phases of trachoma virus as seen in films of conjunctival scrapings derived from the disease.

d. Illustrates the appearance of an histological section of trachomatous tissue. A full description of these inclusions appears in Ch. XLVII, p. 608.

f. Shows an epithelial cell containing a mass of elementary bodies adjacent to the cell nucleus. The illustration indicates the appearance of the inclusion conjunctivitis virus (see Ch. XLVIII).

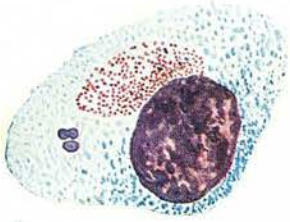
In their morphology, the inclusions of trachoma so closely resemble those found in inclusion conjunctivitis that for purposes of practical diagnosis they may be regarded as identical.

All the above have been stained with Giemsa's solution, and are magnified × 1,200 approximately.

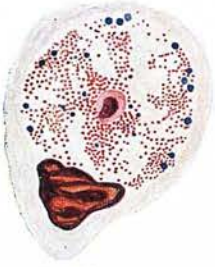
Modified from Halberstaedter (1912). (See Ch. XLVII.)

PLATE IV

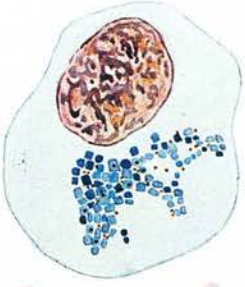
a



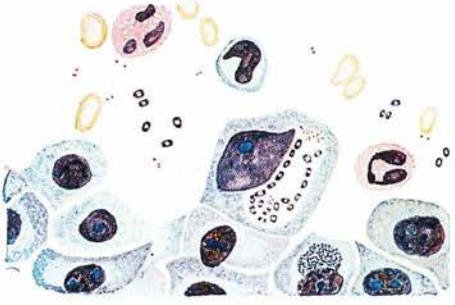
f



b



e



c

d

C.E.v.R. and A.J.R.

gawa's bodies), trachoma, inclusion conjunctivitis, psittacosis, ectromelia of mice, and fowl-pox.

There are, however, certain other types of intracytoplasmic inclusion which have not been so fully studied and may be non-specific, being unconnected with the causal agent. It is possible, however, that further study may remove some of these structures into the group just mentioned above. Bodies, the specificity of which we do not yet regard as established, are: Da Fano's or Nicolau's bodies of herpes febrilis; Da Fano's or Dawson's bodies of encephalitis lethargica; Taniguchi's bodies of Japanese encephalitis; eosinophilic bodies in warts; basophilic bodies in measles.

Now, with regard to intranuclear inclusions the position is rather different. For, first, intranuclear inclusions occur in a variety of non-virus infections, in healthy animals and human beings, and can be produced experimentally. Second, there are only two common appearances presented by intranuclear inclusions—Types A and B. Many nuclear inclusions, however, are found so uniformly in the particular disease, and are so constantly associated with the infectivity of the tissue in which they occur, that they may be regarded as specific structures due to the virus in question. Inclusions which we so regard are those associated with yellow fever, herpes (Lipschütz bodies), Rift Valley fever, varicella, 'B' virus, and virus III. With regard to the intranuclear inclusions of Nicolau in herpes, and the inclusions of poliomyelitis and zoster, we would reserve our opinion until further and more definite reports are received.

To conclude, we would summarize our views on the specificity of inclusion bodies in the following terms: Certain inclusions, both cytoplasmic and nuclear, are so constantly found in virus infections that they must be regarded as specific structures produced by the respective virus agents. There are, however, certain less definite inclusions, which have not been so thoroughly studied. We would not place these in the same category as those just mentioned, and we consider that their presence may be merely fortuitous; more particularly as it is evident that bodies morphologically resembling those of virus diseases can occur in conditions where the presence of any virus is improbable. The mere finding of an inclusion in a cell does not indicate a virus infection; the inclusions must occur constantly in cases of the disease, and be associated with the infectivity of the tissue in which they occur, before the possibility of a virus infection can even be considered.

THE STRUCTURE OF INCLUSIONS

Any discussion on the structure of inclusions at the present day must take note of one outstanding fact. That is, that two typical intracytoplasmic inclusions—those of ectromelia and fowl-pox—have been isolated by microdissection, and found to be infective; further, one typical intranuclear inclusion—the Lipschütz body of herpes—has also been found to be infective.

There are two main theories in regard to the composition of inclusions. In the first place, it is suggested that inclusions are formed from the cells of the host; that is to say, the inclusions are reactionary products. For example, it has been variously suggested that certain inclusions may be formed by a process of degeneration of the cytoplasm or of the nucleus; that they are formed from extruded chromatin or nucleoli; that they are formed from the Nissl substance or mitochondria; or that they are formed from hypertrophied neurofibrils. It is possible that the well-known Negri body is formed from nuclear material, or else from neuro-

fibrils, but we do not believe that many other common types of inclusion can be satisfactorily explained by this theory. In the second place, it has been suggested that inclusions are intracellular colonies of virus elementary bodies. We believe that this theory explains the inclusions of ectromelia, fowl-pox, molluscum contagiosum, variola-vaccinia, psittacosis, trachoma, inclusion conjunctivitis, lymphogranuloma inguinale, and herpes febrilis (Lipschütz bodies). It is probable that the most feasible explanation of the composition of the majority of inclusions is that elementary bodies first enter the cell, sometimes reaching the nucleus, and then proliferate to form a colony. The cell itself probably reacts and produces a covering which gives the inclusion a more or less homogeneous appearance; especially is this so in the trachoma group of inclusions. This theory was really advanced many years ago by von Prowazek (1907), who held that inclusions were formed from living organisms embedded in a matrix produced by the cell. He coined the term *Chlamydozoa* to express the idea of organisms being clothed by a mantle of cellular protoplasmic material. Later, Lipschütz (1921) regarded nuclear inclusions as reactionary products with which were associated virus particles.

It has been stated in our introductory section that viruses may be regarded as showing the most extreme degree of parasitism in nature. The formation of colonies, i.e. inclusions, inside cells is probably necessitated by the limited metabolic powers of this group of agents. If this be so, however, why have not inclusions been demonstrated in all virus infections ? Perhaps it is only because our staining methods are relatively crude and sometimes fail to demonstrate the presence of virus aggregates in cytoplasm or in nucleus.

The problem of the biological nature of inclusions calls for much further study; at the present, too much use has to be made of analogy. More experimental data, especially such as can be yielded by micromanipulation, are much required.

REFERENCES

Acton, H. W., and Harvey, W. F. (1911), *Parasitology*, **4**, 255.
Andrewes, C. H. (1930), *J. Path. Bact.*, **33**, 301.
Baumgartner, G. (1935 *a*), *Zbl. Bakt.*, Abt. I, orig., **133**, 282.
—— (1935 *b*), *Schweiz. med. Wschr.*, **16**, 759.
Bedson, S. P., and Bland, J. O. W. (1932), *Brit. J. exp. Path.*, **13**, 461.
Blackman, S. S. (Jr.) (1936), *Johns Hopk. Hosp. Bull.*, **58**, 384.
Broadhurst, J., Cameron, G., Maclean, M. E., and Saurino, V. (1939), *J. infect. Dis.* **64**, 193.
Covell, W. P. (1932), *Amer. J. Path.*, **8**, 151.
Covell, W. P., and Danks, W. B. C. (1932), *Amer. J. Path.*, **8**, 557.
Cowdry, E. V. (1933), *Amer. J. Path.*, **9**, 149.
—— (1934), *Arch. Path.*, **18**, 527.
Cowdry, E. V., and Scott, G. H. (1930), *Arch. Path.*, **9**, 1184.
—— —— (1935 *a*), *Amer. J. Path.*, **11**, 647.
—— —— (1935 *b*), *Amer. J. Path.*, **11**, 659.
Cowdry, E. V., Lucas, A. M., and Fox, H. (1935), *Amer. J. Path.*, **11**, 237.
Danks, W. B. C. (1932), *Amer. J. Path.*, **8**, 711.
Davenport, H. A., Ranson, S. W., and Terwilliger, E. H. (1931), *Anat. Rec.*, **48**, 251.
Farber, S., and Wolbach, S. B. (1932), *Amer. J. Path.*, **8**, 123.
Findlay, G. M. (1932), *Brit. J. exp. Path.*, **13**, 223.
Findlay, G. M., and Ludford, R. J. (1926), *Brit. J. exp. Path.*, **7**, 223.
Findlay, G. M., Mackenzie, R. D., and MacCallum, F. O. (1938), *Trans. R. Soc. trop. Med. Hyg.*, **32**, 183.
Goodpasture, E. W., Auerbach, S. H., Swanson, H. S., and Cotter, E. F. (1939), *Amer. J. Dis. Child*, **57**, 997.
Goodpasture, E. W., and Talbot, F. B. (1921), *Amer. J. Dis. Child.*, **21**, 415.

Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY
Vol. XLVI., No. 3, pp. 425-436, 1938.

616—006 . 393—093

THE MICROMANIPULATION AND MICRODISSECTION OF THE MOLLUSCUM CONTAGIOSUM INCLUSION BODY.

C. E. VAN ROOYEN.

From the Bacteriology Department, Edinburgh University.

(PLATES XXXVI.-XL.)

THE infectivity of molluscum contagiosum as well as the characteristic inclusion bodies associated with the disease were first described (separately) by Henderson and Paterson of Edinburgh in 1841. Ever since the discovery of these large cell inclusions, many views have been expressed as to their probable ætiological significance.

Bateman (1817), who originally recognised the disease as a clinical entity, believed that the caseous material which exuded from the nodular swellings represented a form of secretion. Virchow (1865) believed that the little swellings were enlarged sebaceous glands, Unna (1896) and others that they arose from cells which had proliferated from the malpighian layer of the epidermis. Lipschütz (1911) studied the Henderson-Paterson inclusion body (molluscum body) and found that it contained countless minute, non-motile, spherical bodies of about 0·25 μ diameter. These he called *Strongyloplasma hominis* and maintained that they were a form of protozoal parasite and responsible for the disease. von Prowazek (1911) also found elementary bodies. Neisser (quoted by Joseph and van Deventer, 1906) made similar observations and came to the same conclusion. Joseph and van Deventer however disbelieved the parasitic theory and regarded the molluscum body as a hyaline or colloid degenerated cell.

The infective agent of molluscum contagiosum has repeatedly been proved to be filterable. Juliusberg (1905) showed that it passed through the Chamberland filter, the filtrate when inoculated into the human subject reproducing the disease after an incubation period of 50 days. Wile and Kingery (1919) demonstrated its ability to pass through the Berkefeld filter and found the incubation period to be 14-25 days. Findlay (1930) succeeded in filtering infective material through the Berkefeld V and Chamberland L1 candles and reported the incubation period to be 5 weeks.

At the present time therefore molluscum contagiosum may be regarded as a typical example of a virus disease. The infective agent is readily filterable, the disease exhibits a distinct incubation period and the lesions in epithelial cells are accompanied by the

formation of large cytoplasmic acidophile inclusion bodies. Moreover the latter have been shown by Lipschütz and by von Prowazek to contain innumerable small granules which are now accepted as typical elementary bodies resembling those found in vaccinia by Buist in 1887 (see Gordon, 1937 ; Mackie and van Rooyen, 1937) and in other virus diseases.

Goodpasture and King (1927) have reinvestigated the development of the molluscum inclusion body by means of stained sections and smear preparations and have arrived at the following conclusions.

> 'The main factor in the hyalinization of the intracellular mass is desiccation. The hyaline oval masses formed by a coalescence of granules and cytoplasm constitute the mature " molluscum bodies " of Henderson and Paterson. They are not formed by a sort of keratinization as suggested by Lipschütz, but by fusion and desiccation of the elementary bodies and the intervening cytoplasm.
>
> 'It is evident from our preparations that the particles which appear to be extruded from the nucleus have no part in the composition of the elementary bodies, but dissolve and become a part of the basophilic cytoplasm which has a central position within the cell. It is within this altered cytoplasm that the vacuoles develop, about which and eventually within which tiny bodies occur having no counterpart among the cellular constituents. Mitochondria play no part in the formation of the elementary bodies. In fact the elementary bodies constitute, from all cytologic appearances, a new substance which increases enormously in bulk in the affected cells, and not by accretions to the size of individual bodies, but by a proliferation of innumerable bodies of uniform size, form, and staining qualities. The cytologic changes are in every way consistent with an active growth of a very minute living microörganism. Smear preparations, made by stroking a glass slide over the pearly core of a lesion moistened with physiologic salt solution, and suitably stained, confirm the cytologic appearances of an intracellular microörganism.'

According to these authors, then, the formation of the molluscum body is effected by fusion and desiccation of the elementary bodies and intervening cytoplasm of the affected cell. The process moreover takes place without the formation of a membrane covering the inclusion body.

Findlay in his excellent and comprehensive review of the literature of this subject, makes the following further reference to the absence of a membrane encircling the molluscum body.

> " The development of these molluscum bodies has recently been reinvestigated by Goodpasture and King (1927), whose description corresponds very closely with the account of the development of the virus inclusions in fowl-pox given by Ludford and Findlay (1926), except that in human molluscum contagiosum the virus vacuoles do not acquire a lipoid coat."

The present investigation.

The work was carried out with material obtained from ten cases of molluscum contagiosum under treatment by Drs Percival and

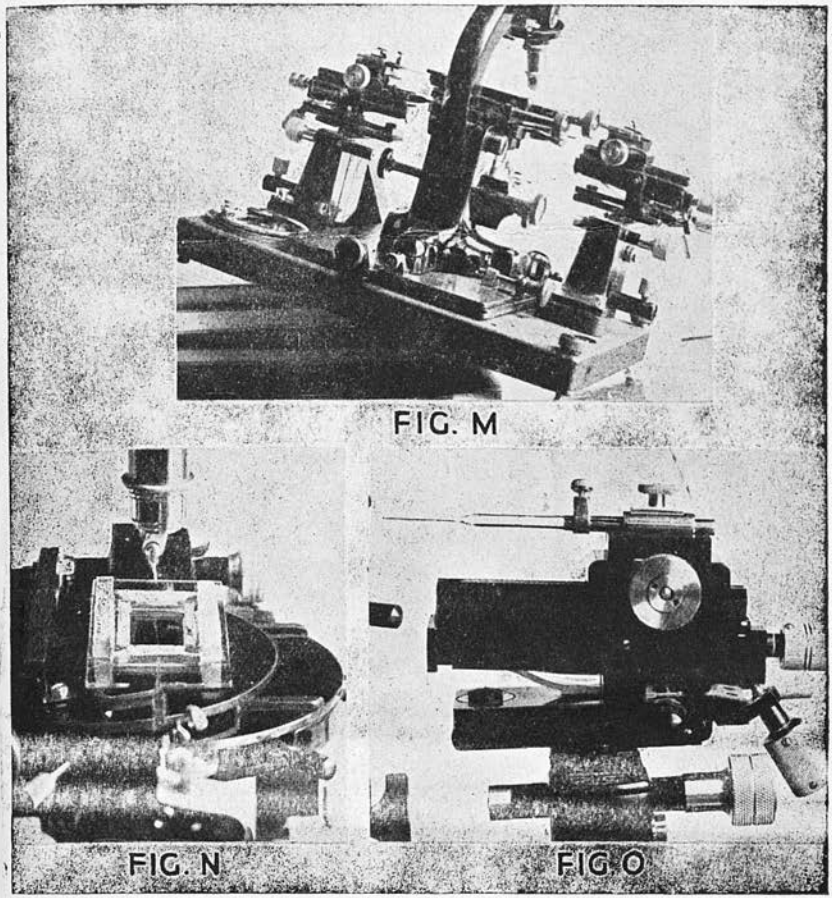


FIG. N. A view of the dissecting chamber from above, cover-slip removed.

FIG. M. Microscope tilted at an angle in order to display the two adjustment screws of the foot-plate centring device.

FIG. O. Shows the dissecting needle clamped in its holder and secured to the manipulator on the right-hand side.

Peterkin of the Dermatological Department of the Royal Infirmary of Edinburgh. The freshly obtained material was stained with vital dyes and the cells subjected to microdissection with fine glass needles. By micromanipulation of living tissues, it has been possible to detect certain morphological features and details of structure which are unappreciable in fixed and stained preparations (fig. 1).

In a short publication such as this, it is impracticable to give a full account of the design of micromanipulator used or the methods by which the needles were fashioned and the instrument operated. For fuller details reference should be made to one of the standard treatises dealing with the theory and practice of micromanipulation (Chambers, 1918, 1921, 1922*a*, 1922*b*, 1922-23, 1923 ; Péterfi, 1928 ; Howland and Belkin, 1931 ; Schouten, 1936).

The following account summarises the principal features of the apparatus employed by the writer, including details of accessories designed by himself to meet his own requirements.

Details of instrument.

The micromanipulator. Standard Janse and Péterfi (Zeiss) micromanipulating instrument, comprising right and left hand operating stands. Each of the latter was fitted with 7 adjustments, 4 coarse and 3 fine, so that the point of the glass needle attached to each arm was capable of moving vertically, horizontally and in the arc of a circle. Each glass dissecting needle was mounted on a specially designed metal holder which was fastened to the actuating limb of the manipulator. The holder consisted of a cylindrical brass rod 7·5 mm. in diameter and 20 mm. in length, through which a hole was drilled in an eccentric position. A piece of brass tubing was now fitted into this hole and welded into position and the glass dissecting needles were inserted into the lumen of the brass tubing and secured with a clamp. With metal needle holders such as these it was possible to set the needle at any desired length as well as to rotate the needle point through a complete circle. The radius of the latter varied with different needles but was generally about 1·5 mm. in diameter.

The microscope. Watson's Patna instrument fitted with a universal stage permitting rotating and eccentric movements, rack-work draw tube, and centring nosepiece for objectives. *Substage equipment.* Centring substage with coarse and fine adjustments, iris diaphragm and Leitz long-focus condenser possessing focal intercepts of 15 mm. *Objectives.* Watson $\frac{2}{3}$ in., Leitz 6L and Zeiss 8 mm. apochromatic, and Watson 2 mm. holoscopic objectives. *Eyepieces.* Watson holoscopic ×10 and ×20, Zeiss ×12 and ×25 and Huyghenian ×8 and ×10 eyepieces.

Microscope foot-plate-centring device. This was devised in order to afford a method by which the whole microscope could be moved either horizontally or vertically without altering the position of the dissecting needles relative to each other or to the field above them. The instrument was constructed of two smooth plain metal plates 19 cm. long, 17 cm. broad and 4 mm. thick which moved over each other on ball-bearing surfaces. The lower plate was secured to the dissecting instrument bedplate, the upper movable part to the foot plate of the microscope, and the motions of the upper half were controlled by means of milled-headed metal screws. This device made it

possible to bring the optical axis of the microscope over the dissecting needles with ease. Thus both the needle points could be centred simultaneously without touching the micromanipulating stands, or moving the film of cells (above the needles) placed on the dissecting chamber coverslip. The latter was controlled by the mechanical stage.

See fig. L.

Dissecting chamber. This was constructed from glass according to the author's own requirement and was not of standard pattern. It consisted of a glass slide 76 mm. long, 37 mm. wide and 1·1 mm. thick, on which was cemented together a rectangular glass chamber 20 × 30 mm. constructed of two sides 35 mm. long, 7 mm. deep and 4 mm. wide and two lateral walls 20 mm. long, 2·5 mm. deep and 2·5 mm. in width.

Two glass " troughs " were also provided along the lateral border of the chamber into which water was placed in order to maintain a sufficient degree of moisture within the chamber during dissection.

Technique.

Glass coverslips 38 mm. long, 31 mm. broad and 0·14 mm. thick were cleaned by boiling in a mixture of potassium bichromate solution and sulphuric acid, washed with water, placed in methylated spirit for 1 hour, removed and dried with a silk cloth. The surface of the coverslip was then treated with a few flakes of trilaurine which was rubbed into the glass with an artist's paper pencil. The coverslip was subsequently passed rapidly through the bunsen flame three times, placed on the laboratory bench and the excess of grease wiped off its surface with a silk rag.

A fresh moist fragment of molluscum tissue about 1 mm. in diameter was now placed at one end of the coverslip and uniformly spread over the surface by crushing it with the edge of a 3 in. × 1 in. glass slide.

While the smear was still moist one drop of a 1 : 10,000 dilution of brilliant cresyl blue in 0·86 per cent. saline was placed on the film and gently allowed to flow over it. The coverslip was next picked off the laboratory bench and the film placed inverted over the dissecting chamber. Next both manipulating needles were accurately centred in the field so as to take up a position slightly below the level of the film without actually touching it. Both lateral openings through which the needles passed into the dissecting chamber were now sealed up with vaseline and the interior of the cavity made air-tight. Any minor adjustments to the position of the substage condenser, iris diaphragm and reflecting mirror were made at the same time.

Prior to commencing micromanipulation the $\frac{2}{3}$ in. objective and × 10 ocular were placed in position, the light adjusted to maximum intensity and a suitably stained group of molluscum bodies manœuvred into the centre of the field. At this stage both dissecting needles should be lying about 250 μ below the level of the film and should not be permitted to establish contact with the wet surface of the film. The needles were next elevated vertically until the point of each became just recognisable as a blurred image in the field.

To facilitate the latter operation the N.A. of the condenser was decreased by closing the iris diaphragm slightly, in order to give greater depth of focus and to render both needle points and cells lying above them more easily visible. The needle points, which should be close to each other, were re-centred in the field and the cell or group of cells to be dissected moved so that they lay immediately above the needles. The 6L (Leitz) objective and × 10 eyepiece (total magnification 450) were now substituted for the others and the dissecting needles gently raised and adjusted so that one needle lay immediately beneath the cytoplasm and the other below the inclusion body (fig. 2). A drop of cedar-wood oil was placed on the coverslip,

FIG. L.

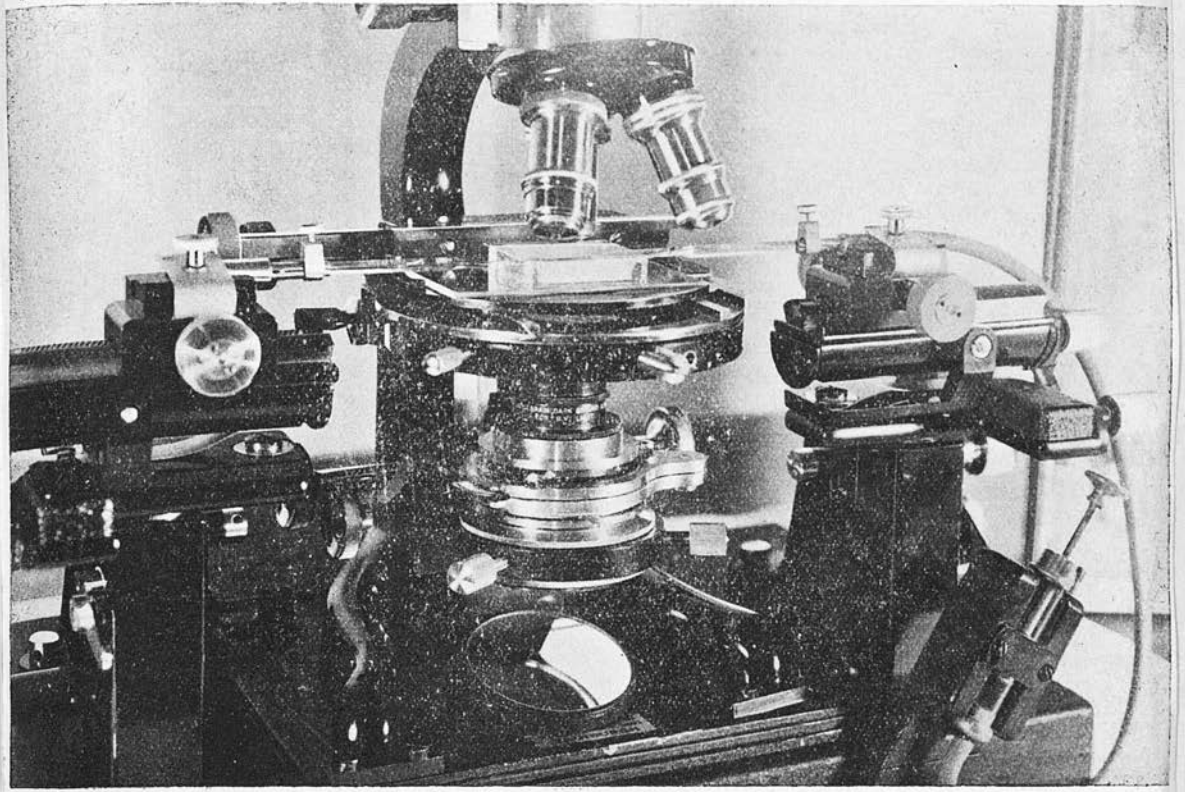


FIG. L. Shows the general lay-out of the components illustrated in Figs. M, N and O, and a view of the equipment employed by the authors.

the 2 mm. holoscopic oil immersion objective and ×10 holoscopic (Watson) compensating ocular placed in position, and the cell, inclusion body and two needles below were re-focussed and re-centred if necessary. The needle below the cytoplasm was now gently elevated so that it first touched the cytoplasm, next firmly pressed it against the coverslip and finally penetrated it (fig. 2). A little lateral movement of the needle tended to facilitate the final step of the last manœuvre. The cell wall was now firmly impinged against the glass coverslip and made immovably fast so that the work of tearing the inclusion body out of the cell could be proceeded with. The needle lying below the inclusion body was elevated and manœuvred as previously so that the point of the needle was first made to incise the cytoplasm covering the inclusion body (fig. 3), and later to come into contact with its under side. By gentle traction the inclusion body was extracted from the cytoplasm (fig. 4), the line of force lying in a direction opposite to that exerted by the " steadying " needle. Before removing the molluscum body from the cell the position of the nucleus was determined if possible. This usually appeared as a vacuolated, degenerate structure huddled against one side of the cell wall, but in many of the infected cells no nucleus could be seen.

Observations on the cytoplasm of the infected epithelial cell.

Observations under 1000 magnification suggested that the molluscum body occupied a potential cavity within the cell (fig. 5). After its removal there was no tendency for the protoplasm to flow into this hollow space, which appeared to be surrounded by a " wall " of condensed protoplasm. Moreover it was possible to insert a blunt needle point into the cavity and to explore its boundaries, but it was never possible to show the presence of a definite lining wall or membrane. The wall of the cavity, however, could be punctured with a sharp needle and the protoplasm then flowed into it through the artificially created communication. Measurements of the cavity (fig. 6) and of the inclusion body extracted from it showed that they were almost the same size. For example in the case of an oblong epithelial cell measuring 60×45 μ and containing an oval molluscum body of 30-35 μ diameter (fig. 7), the cavity left after removal of the latter was found to be approximately 36-37 μ in diameter. Only rough measurements of the size of the inclusion body cavity were practicable, because this hollow socket, being curved, possessed several dimensions and the figures stated could only be regarded as approximate.

These observations were repeated on 25 different cells derived from 10 cases of molluscum contagiosum. On each occasion it was possible to show that a large hollow cavity remained in the cell after the molluscum body had been removed. Occasionally it was possible to extract the inclusion body from the cell with the tip of the needle, replace it in the cavity and again remove it without either damaging the cell structure or destroying the inclusion body cavity. The existence of this hollow cavity was further demonstrated by the injection into it of indian ink particles and by means of dark-ground illumination.

Injection of carbon particles into the inclusion cavity.

The indian ink used was first filtered and then centrifuged at 3000 *r.p.m.* for 30 minutes. After withdrawing the inclusion body from an epithelial cell, the sharp-pointed needle was discarded and there was substituted a hollow glass pipette possessing a lumen of about 2 μ and an external diameter of about 2·4 μ. The pipette, filled with indian ink, was inserted into the cavity and the ink discharged into it. By this method it was possible to fill the cavity with carbon particles and to demonstrate that there was no tendency for them to flow into the adjacent cytoplasm. By rupturing the cavity wall, however, the ink was made to flow into it. The boundaries of the cavity were also delineated by injecting indian ink into the cytoplasm so that the ink outlined the margins of the cavity but did not enter it—unless it was punctured with a needle point either before or after introducing the ink.

Observations with dark-ground illumination.

When an epithelial cell containing an inclusion body was examined under dark-ground illumination, both the cytoplasm and the molluscum body appeared to be full of refractile particles, but when the inclusion was removed from the cell, a vacant semi-translucent space was left where previously the inclusion body had lain. Again there was no evidence of a lining membrane.

The refractile granules within the cytoplasm were closely compared with those inside the molluscum body and a number of significant differences were detected. From their histological studies Goodpasture and King concluded that the epithelial cell contains myriads of elementary bodies measuring about 0·25 μ in diameter. The writer, however, believes that the majority of the refractile particles evident within the cytoplasm of the epithelial cell are probably minute vacuoles or protoplasmic granules and not true elementary bodies. His reasons for this assumption are the fact that the refractile particles in the cytoplasm range from 0·5 to 3·5 μ in diameter, whereas the stained molluscum elementary body is remarkably uniform in size—0·3 μ by mensuration and 0·35 μ when estimated by micrometric extinction (van Rooyen, 1937). By exerting pressure on the cell and exploring its contents by inserting a needle, many of the refractile spots in the cytoplasm could be shown to be minute spaces surrounded by concentric diffraction rings.

Similar examination of the molluscum inclusion body showed that its structure was entirely different, for it contained numerous refractile particles which in shape, size and degree of refractility were identical with typical virus elementary bodies (fig. 11). Some additional evidence in support of this contention was derived from

MICRODISSECTION OF MOLLUSCUM BODY

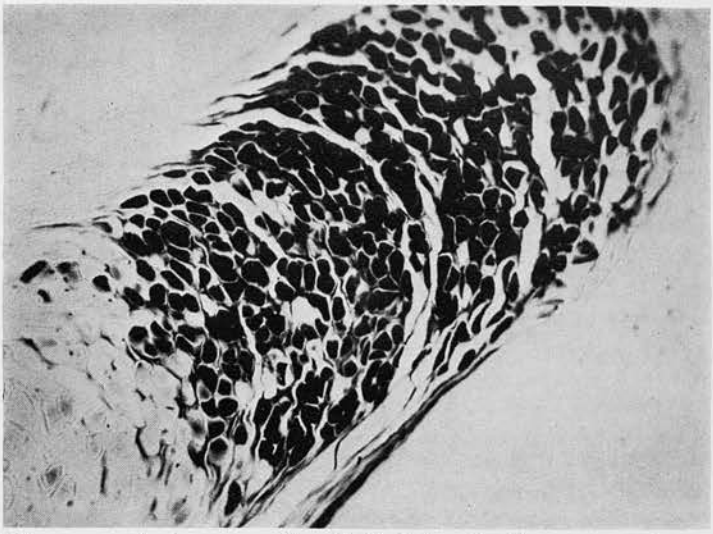


FIG. 1.—Molluscum contagiosum: section of skin lesion showing numerous intracytoplasmic inclusion bodies which appear black. Mann's stain. ×600.

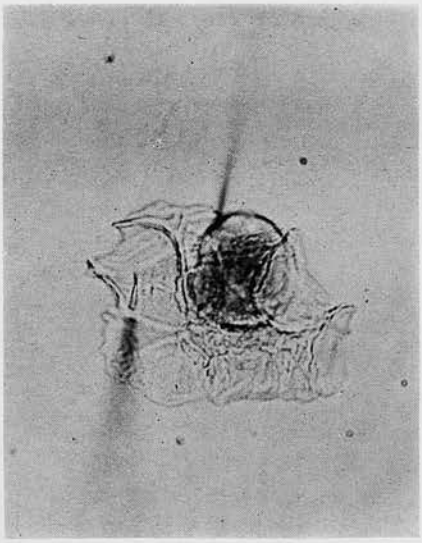


FIG. 2.—Photomicrograph of an infected epithelial cell containing a large molluscum body. The cell is about to be microdissected; the lower needle point has been inserted into the cytoplasm in order to steady the cell, the other needle is lying below the molluscum body. It occupies a lower plane and is therefore not clearly visible. ×700.

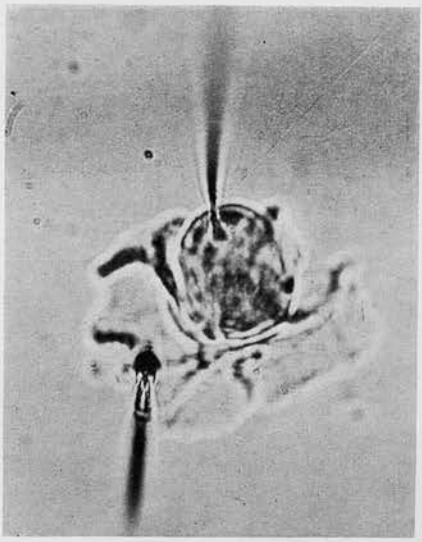


FIG. 3.—Both dissecting needles are now in focus, one being placed in the cytoplasm, the other in the inclusion body. Slight traction has been exerted upon the molluscum body, which has moved its position slightly. ×700.

MICRODISSECTION OF MOLLUSCUM BODY

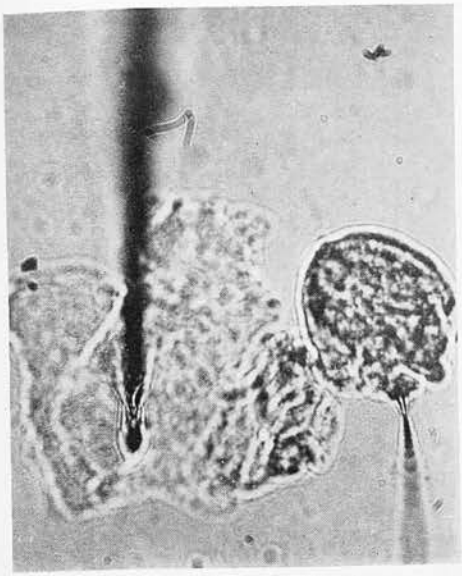


FIG. 4.—Molluscum body (right) after removal from the cell (left). ×700.

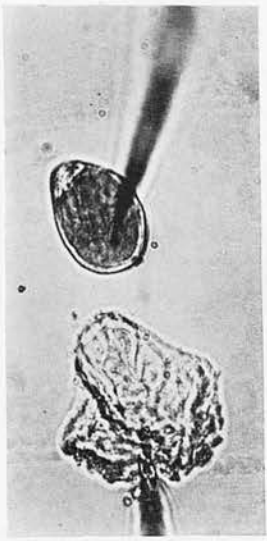


FIG. 5.—Photomicrograph taken shortly after the removal, by microdissection, of a molluscum body from an epithelial cell. Note the pear-shaped contour of the body, the membrane surrounding it and the thin-walled "cap" at the upper pole. Also observe suggestion of a cavity in the cell. ×500.

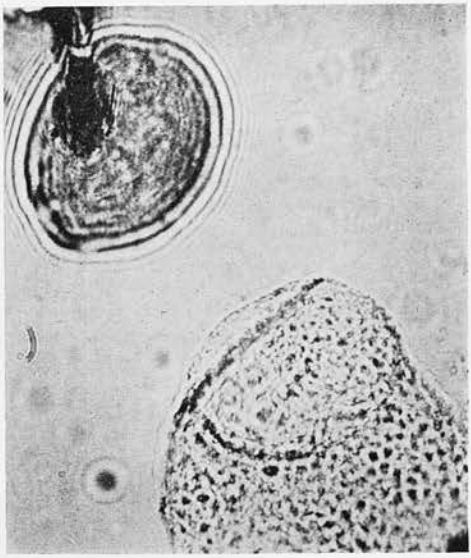


FIG. 6.—Higher magnification, showing the cytoplasm of the epithelial cell accurately in focus, clearly revealing the oval cavity from which the molluscum body has been extracted. The body itself is lying at a lower focal plane and being out of focus is surrounded by diffraction rings. ×1000.

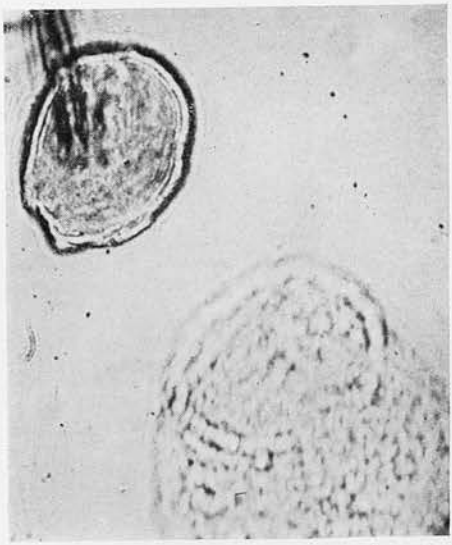


FIG. 7.—Similar to fig. 6, but showing the molluscum body carefully focussed, the cell out of focus and now lying in a higher optical plane. Note pear-shaped outline of the molluscum body, with broad, thick-walled segment above and thin-walled projecting pole below. ×1000.

MICRODISSECTION OF MOLLUSCUM BODY

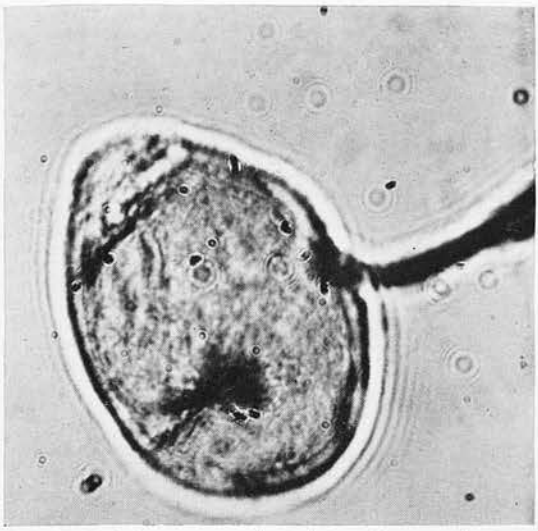


FIG. 8.—Higher power view of a molluscum body on the tip of the dissecting needle. Observe the translucent pole (above) and the more opaque, denser lower portion. ×1680.

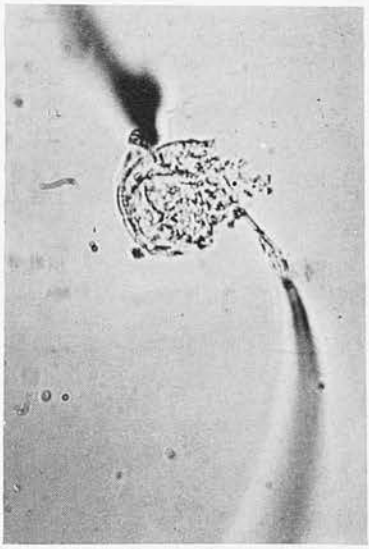


FIG. 9. — Photomicrograph of a molluscum body taken immediately after its membrane was incised with the dissecting needle. Note commencing diffusion of small fragments into surrounding fluid. ×500.

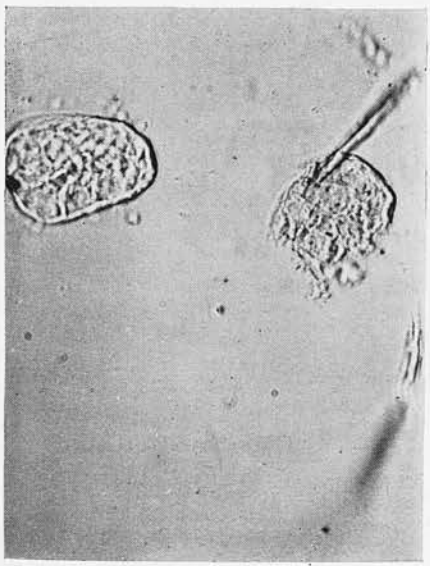


FIG. 10.—Similar to fig. 9, but taken at a slightly different level of focus. Note undissected molluscum body alongside. ×500.

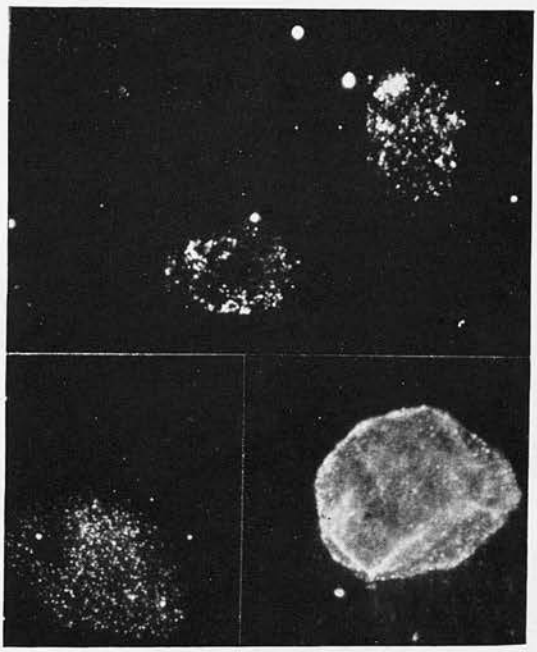


FIG. 11.—Photomicrograph of 4 molluscum inclusion bodies taken with dark-ground illumination. Observe the highly refractile granules (virus bodies) within them. From a fresh, wet, unfixed and unstained preparation. ×600.

Plate XXXIX

Fig. 12.—Film from a molluscum lesion stained by Paschen's method for elementary bodies. Observe the enormous number of these minute virus bodies, also the large molluscum inclusion body in the left upper quadrant. ×1200.

Fig. 13.—Effect of compressing a molluscum body with a micro-needle against a coverslip. The needle is pointing obliquely upwards. The contents tend to herniate through the conical pole, thus revealing its structural weakness. ×1100.

Fig. 14.—Demonstrates the mechanical strength of the lateral walls of the molluscum body by compressing it between the sharp point of a needle and a flat micro-spatula. Note the lighter staining "cap" or tapering pole. ×1100.

Fig. 15.—Reveals the ease with which the needle point can be inserted into the interior of the molluscum body through the weak conical pole. The membrane at this point offers no resistance to the pressure of the needle. ×1100.

Fig. 16.—Two micro-needles have transfixed a molluscum body at either extremity and then been pulled apart. Observe the low tensile strength of the thin wall of the conical pole and its tendency to break. ×1100.

MICRODISSECTION OF MOLLUSCUM BODY

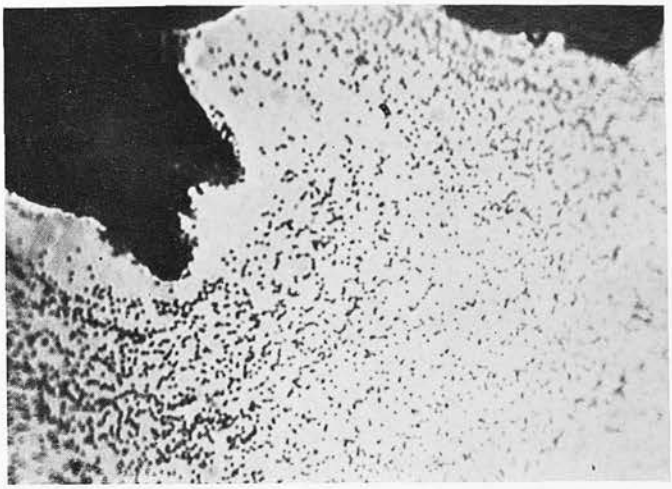


FIG. 12.

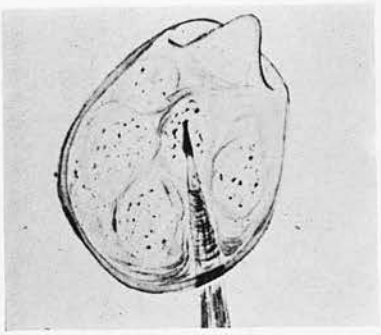


FIG. 13.

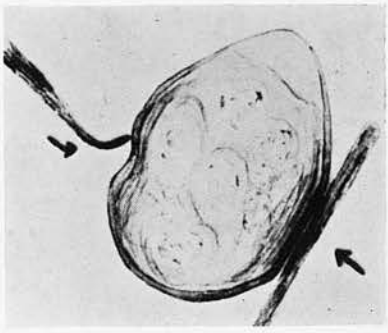


FIG. 14.

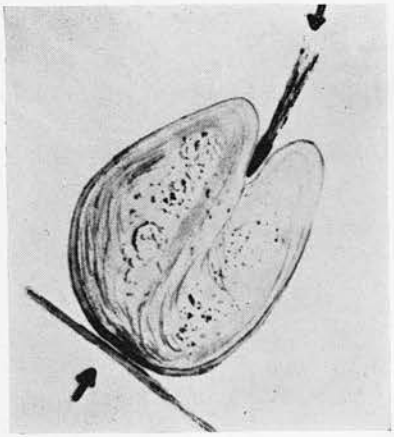


FIG. 15.

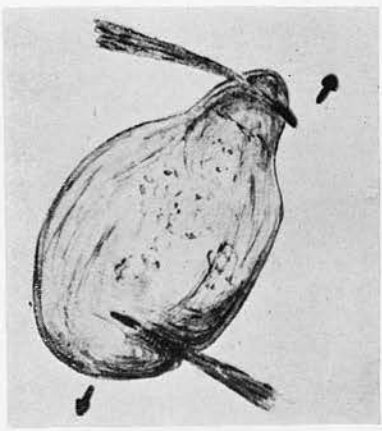


FIG 16.

MICRODISSECTION OF MOLLUSCUM BODY

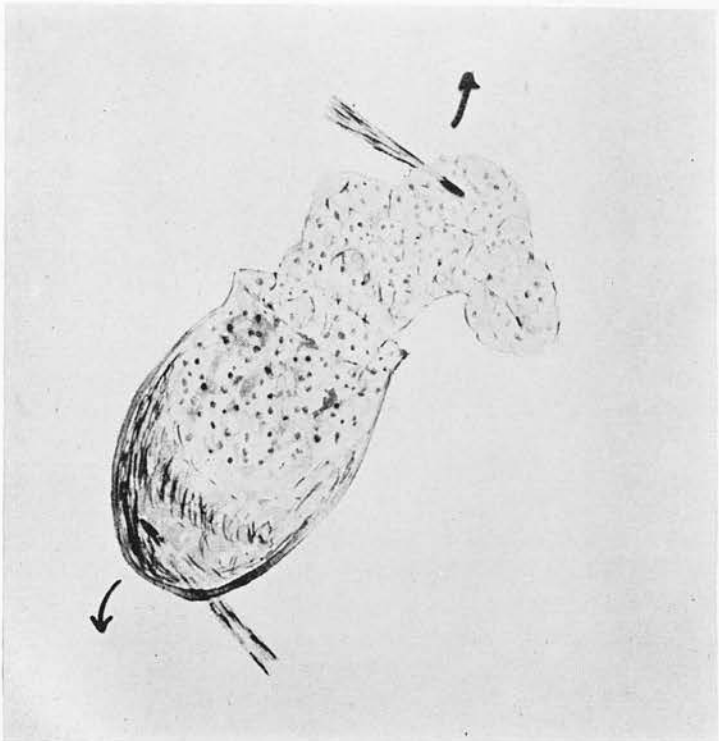


FIG. 17.—Shows the internal contents of a molluscum body in process of removal with the point of a dissecting needle. The contained material tends to be mucoid in consistency and adheres to the point of the needle. The minute elementary bodies are only just visible in the gelatinous matrix surrounding them. After removing this jelly-like substance, the empty "shell" of the molluscum body loses its former segmented internal appearance. ×1100.

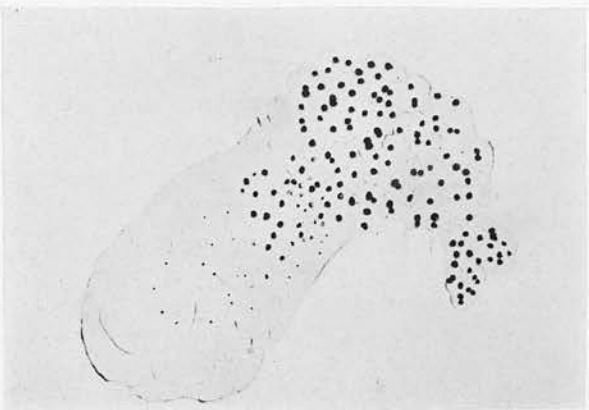


FIG. 18.—Film stained by Paschen's method, showing numerous molluscum elementary bodies which have been liberated from a ruptured inclusion body. ×1100.

observations made on films prepared from molluscum lesions and stained by Paschen's method for elementary bodies (fig. 12). These showed that the majority of the elementary bodies were distributed in the vicinity of ruptured molluscum inclusion bodies, whereas they were often absent in the neighbourhood of fragmented epithelial cells.

To summarise, the molluscum inclusion body appears to be a structure entirely independent of the cytoplasm of the infected epithelial cell in which it lies. It can be freely withdrawn from the cell, leaving a cavity of corresponding size, and it does not seem to be attached or adherent in any way to the cell protoplasm.

Dissection of the molluscum body.

Having removed the inclusion body from the cell with the point of the needle (fig. 7) the other needle (which was placed in the cytoplasm of the cell) was withdrawn from it and also inserted into the molluscum body. Before commencing dissection of the inclusion bodies, 20 of them were measured. The smallest forms tended to be circular, varied from 9 to 24 μ in diameter, and were surrounded by a wall of uniform thickness which usually measured from 0·5 to 0·75 μ in width. The largest bodies were oval or pear shaped and varied from 24 to 27 μ in width and from 30 to 37 μ in length. They were each surrounded by a membrane which was about 0·7 μ thick at the broad pole of the body and 0·15 μ thick at its conical pole (fig. 7). Extreme care was taken in making these measurements to eliminate the possibility of optical error. Thus every possible precaution was taken to ensure conditions of critical microscopy such as uniform lighting of the field, accurate centring of objectives, condenser and lamp filament and use of the optimum draw-tube length.

When examined under a comparatively low power magnification the molluscum body appears as a hyaline, homogeneous, red-staining body devoid of any particular internal structure. When fresh molluscum bodies were stained with a vital dye, however, such as brilliant cresyl blue, a definite structure could be seen. Observations on the undissected inclusion body suggested that it contained internally a portion occupying about three-quarters of its length filled with densely staining material and a thin-walled, conical, more lightly stained segment or " cap " (fig. 8), the existence of which could be demonstrated in the following ways.*

1. When the short axis of the inclusion body was compressed

* For these observations a good 2 mm. apochromatic oil immersion objective and ×20 compensating ocular were used in conjunction with a short filament, low voltage and high intensity source of illumination ; a light blue or green ground glass filter was also employed with advantage. During the course of these observations the substage condenser diaphragm was closed slightly in order to provide greater depth of focus.

against the flattened edge of a micro-spatula and a sharp needle was pushed into the opposite side the needle bent before puncturing the cell (fig. 14).

2. The rounded, blunt extremity of the inclusion body appeared to have the thickest wall and when the cell was compressed in its long axis, the thin-walled cap tended to flatten out against the micro-spatula as pressure was applied with the needle from the blunt end: thereafter the needle point bent and finally the cell wall yielded inwards.

3. When this procedure was reversed and the flat edge of the micro-spatula was applied to the blunt extremity and the needle point placed against the cap a different sequence of events was observed when force was exerted. The needle point immediately and easily penetrated the thin cap of the inclusion body whilst the thicker, broader segment remained firm and rigid against the face of the spatula (fig. 15).

4. If the inclusion body was picked up between the points of two needles and allowed to rotate between them the presence of the cap could be detected as the inclusion body altered its axis in the field.

5. By compressing the inclusion body against the under side of the coverslip with a broad needle about 10 μ in diameter the cap could be caused to bulge (fig. 13) and finally to burst. By gradually increasing the pressure the contents of the body were forced out through the breach.

6. When the inclusion body was transfixed at either pole with a sharp pair of needles and then pulled apart (fig. 16) the low tensile strength of the cap was immediately apparent. Shortly before the body was torn asunder it was noticed that the line of cleavage first appeared at the conical pole and later the cell threatened to divide at its uppermost portion. The membrane of the lower (broader) segment tended to remain rigid and usually resisted tearing when subjected to stretching in this manner.

Thus after a series of optical and mechanical tests performed on the molluscum inclusion body the evidence proved the existence of thin-walled and thicker-walled poles. It should be clearly understood, however, that this only applies to bodies which were oval or pyriform in contour. The smaller circular inclusions appeared to possess a wall of uniform thickness. There is no evidence to show whether the size and shape of these bodies is related to their age and degree of development, and if so, which of the two represents the later stage of growth.

The contents of the molluscum body.

Before incising the wall of the inclusion body it was photographed by dark-ground illumination and found to contain numerous

refractile bodies closely resembling in shape, size and degree of refractility the Paschen bodies of vaccinia and other virus diseases. These elementary bodies were not easily photographed *in situ*, for the surface of the living molluscum body is curved, it possesses a membrane of varying thickness and, as will be referred to later, the elementary bodies are embedded in a fluid matrix. Fig. 11 shows four molluscum bodies containing elementary bodies in their interior. These are necessarily indistinct because the elementary bodies lying immediately above and below the optimum plane of focus tend to introduce shadows into the field and impair the crispness of the images in focus. When stained by intra-vitam dyes such as brilliant cresyl blue, the molluscum body presented a mottled appearance as though it were segmented internally into a series of compartments. von Prowazek (1911) and Goodpasture and King (1927) have depicted this appearance but make no suggestion as to its nature. Later an explanation of this appearance will be offered.

The dissection of the molluscum body was proceeded with by first transfixing it with one sharp needle and then incising the cell membrane with the other (figs. 9 and 10). The body cavity was then explored by passing the " tearing " needle into its interior and manœuvring it in such a manner as to facilitate the escape of the contents. Careful examination of the escaping material showed it to consist of innumerable elementary bodies, apparently held together by a gelatinous, mucoid material which slowly diffused throughout the surrounding fluid (figs. 17 and 18). This jelly-like material readily adhered to the needle points.

The markings on the molluscum inclusion body were therefore believed to be due to an optical effect, the result of the passage of light through a globoid, semi-translucent envelope filled with viscid fluid throughout which numerous dense, highly refractile particles were dispersed. The appearance of compartments and whorls arose, in the writer's opinion, from varying degrees of density in the gelatinous fluid within the inclusion body and conceivably also from the effect of unevenly dispersed elementary bodies within that material.

Fig. 12 shows a smear made from a molluscum lesion stained by Paschen's method for elementary bodies. Numerous elementary bodies are seen in close proximity to a ruptured molluscum inclusion body.

Discussion.

The inclusion body of molluscum contagiosum is generally regarded as a typical example of the inclusion bodies found in virus diseases, but from the present studies it would be incorrect to assume that this is so. The methods of investigation here described have not yet been extended to other virus diseases and until this

is done, the present findings must necessarily refer only to the molluscum inclusion body. It remains to be seen whether or not further micromanipulation studies will reveal that the structure of other virus inclusions, such as the Bollinger, Guarnieri and Marchal bodies, is identical with that of the molluscum body.

Goodpasture and Woodruff (1931) have already carried out comparative micromanipulation studies on the Bollinger body and the molluscum body. They found that the molluscum body could be freed from the surrounding epithelial cells by tryptic digestion, but that it then became sticky and gelatinous so that it could not be manipulated readily with the Chambers microdissection apparatus.

The writer has likewise failed to microdissect with the Chambers apparatus molluscum inclusion bodies which had first been digested with trypsin. By means of the Janse-Péterfi type of instrument, however, and the use of fresh tissues stained *intra vitam* with brilliant cresyl blue, it was possible to examine the molluscum body under natural conditions and to preserve those finer morphological details which are unavoidably lost in digested tissues. This work has confirmed earlier knowledge of the molluscum body with regard to the elementary bodies contained within it and has also drawn attention to additional facts concerning its structure. Thus it has been shown that the inclusion body can be extracted from the epithelial cell without disintegration of the latter. There is no evidence of any connection between the two and the lining of the cavity which remains appears to be formed by a localised protoplasmic condensation which can be punctured and broken down with a needle.

Goodpasture and King have suggested that the main factor responsible for the production of the molluscum body is desiccation and fusion of smaller vacuoles within the cytoplasm of the infected cell. The writer can find no evidence which supports this view.

Indeed there is no evidence that the cytoplasm in any way participates in the composition of the molluscum body. About one hundred inclusion bodies have been examined, and every one from the smallest to the largest has appeared as a totally independent structure within the cell. The writer believes that the inclusion grows from the elementary body after it enters the cell. He does not believe that it is formed from the vacuoles so commonly seen in the parasitised epithelial cell. These are more probably formed as a result of degenerative changes due to interference with cell metabolism by the growth of the parasite within it. It is also conceivable that many of these vacuoles may represent elementary bodies which have been destroyed by intracellular ferments.

The pear-shaped contour of most of the molluscum bodies and the semi-translucent " cap " at the conical extremity are of considerable interest. This cap is demonstrably the thinnest part

of the body wall and rupture with liberation of the contained elementary bodies can be more easily effected here than at any other point.

It is suggested that the large pear-shaped molluscum body represents the most fully advanced developmental stage of the virus, in contradistinction to the smaller spherical inclusions which have a membrane of uniform thickness surrounding them.

Whether or not the possession of a thin-walled conical pole should entitle the molluscum inclusion body to be placed in a category separate from the inclusions of other viruses cannot be determined. From the evidence presented it is interesting to record that in some respects, in miniature, the molluscum body resembles the general structure of a fungus sporangium. Further work on this aspect of the subject is in progress.

Conclusions.

1. Fresh material obtained from ten cases of human molluscum contagiosum has been stained *intra vitam* with brilliant cresyl blue and subjected to microdissection.

2. The molluscum inclusion body has been successfully removed from the infected epithelial cell. It has also been replaced in the same cell without injuring the latter.

3. The removal of the molluscum body leaves a large cavity in the cell. The cytoplasm does not flow into this space, the cavity being apparently lined by a localised condensed layer of cytoplasm.

4. The boundaries of this cavity have been defined by injecting indian ink into it or alternatively by introducing indian ink into the surrounding cytoplasm.

5. About one hundred molluscum bodies from the smallest to the largest have been removed from the containing epithelial cells by microdissection and in no case was there any evidence of attachment between body and cytoplasm.

6. The molluscum body probably grows from a more minute form of the virus. It is not formed by desiccation nor by fusion of any intracytoplasmic constituents of the cell.

7. It is suggested that the large oval or pear-shaped forms of the molluscum body represent a later stage of development than the smaller spherical ones.

8. The large pear-shaped inclusions have been shown to possess a definite outer membrane which is thinnest at the conical pole. These observations have been established by demonstrating the tensile strength of the membrane on " palpation " with the point of a micro-needle. The weakness of the conical segment has also been revealed by transfixing the inclusion body between two needles and exerting traction on its walls.

9. The elementary virus bodies contained within the molluscum inclusion body have been shown to be supported in a gelatinous mucoid substance.

I desire to express my indebtedness to Professor Mackie, at whose instigation this research was carried out and to whom I am indebted for much constructive criticism and help. The work has been done with the aid of an expenses grant from the Medical Research Council. The cost of the expensive apparatus was defrayed by a grant from the Sir Halley Stewart Trust of London.

REFERENCES.

BATEMAN, T.	1817.	Delineations of cutaneous diseases, *London*, plate 61.
BUIST, J. B.	1887.	Vaccinia and variola: a study of their life history, *London*, p. 66.
CHAMBERS, R.	1918.	*Biol. Bull.*, xxxiv. 121.
,, ,, ...	1921.	*Science*, liv. 411, 552.
,, ,, ...	1922*a*.	*J. Roy. Micr. Soc.*, p. 373.
,, ,, ...	1922*b*.	*J. Infect. Dis.*, xxxi. 334.
,, ,, ...	1922-23.	*Anat. Rec.*, xxiv. 1.
,, ,, ...	1923.	*J. Bact.*, viii. 1.
FINDLAY, G. M.	1930.	Medical Research Council, A system of bacteriology, *London*, vii. 248.
GOODPASTURE, E. W., AND KING, H.	1927.	*Amer. J. Path.*, iii. 385.
GOODPASTURE, E. W., AND WOODRUFF, C. E.	1931.	*Ibid.*, vii. 1.
GORDON, M. H.	1937.	*Edinb. Med. J.*, xliv. 65.
HENDERSON, W.	1841.	*Edinb. Med. Surg. J.*, lvi. 213.
HOWLAND, R. B., AND BELKIN, M.	1931.	Manual of micrurgy, *New York*, p. 2.
JOSEPH, M., AND VAN DEVENTER, J. B.	1906.	Atlas of cutaneous morbid histology, *London*, p. 22.
JULIUSBERG, M.	1905.	*Dtsch. med. Wschr.*, xxxi. 1598.
LIPSCHÜTZ, B.	1911.	*Arch. Dermat. Syph., Wien*, cvii. 387.
LUDFORD, R. J., AND FINDLAY, G. M.	1926.	*Brit. J. Exp. Path.*, vii. 256.
MACKIE, T. J., AND VAN ROOYEN, C. E.	1937.	*Edinb. Med. J.*, xliv. 72.
PATERSON, R.	1841.	*Edinb. Med. Surg. J.*, lvi. 279.
PÉTERFI, T.	1928.	Mikrurgische Methodik, *from* Abderhalden's Handbuch der biologischen Arbeitsmethoden, Abt. V., Teil 2, Hälfte I., S. 479, *Berlin*.
VON PROWAZEK, S. . . .	1911.	*Arch. Schiffs- u. Tropen-Hyg.*, xv. 173.
VAN ROOYEN, C. E. . . .	1937.	*Zbl. Bakt.*, Abt. I., Orig., cxxxix. 130.
SCHOUTEN, S. L.	1936.	De Micromanipulator, *Amsterdam*.
UNNA, P. G.	1896.	Histopathology of diseases of the skin, *Edinburgh*, p. 793.
VIRCHOW, R.	1865.	*Arch. path. Anat.*, xxxiii. 144.
WILE, U. J., AND KINGERY, L. B.	1919.	*J. Cutan. Dis.*, xxxvii. 431.

PRINTED IN GREAT BRITAIN BY OLIVER AND BOYD LTD., EDINBURGH

Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY
Vol. XLIV., No. 2, pp. 357-363, 1937

Some Cytological Features of Vaccinial Keratitis in the Rabbit

BY

A. J. RHODES AND C. E. VAN ROOYEN

From the Department of Bacteriology, Edinburgh University

Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY
Vol. XLIV., No. 2, pp. 357-363, 1937

617 . 713—002—018 . 1 : 616 . 913

SOME CYTOLOGICAL FEATURES OF VACCINIAL KERATITIS IN THE RABBIT.

A. J. RHODES and C. E. VAN ROOYEN.

From the Department of Bacteriology, Edinburgh University.

(PLATE XXXII.)

THE morbid histological appearances following inoculation of the rabbit's cornea with vaccinia virus have been described by several workers.

Guarnieri (1892) drew attention to the existence of acidophilic inclusion bodies within the epithelial cells of the cornea. His work has been confirmed by von Prowazek and Miyaji (1914-15), Ungermann and Zuelzer (1920) and Paul (1914-15, 1917-18), who utilised the pathogenicity of human variolous material for the rabbit cornea as the basis of a test for the differential diagnosis of variola from varicella. Later Goodpasture, Woodruff and Buddingh (1932) investigated the nature of these inclusions and suggested that they were composed of an aggregation of smaller particles known as elementary or Paschen bodies, which represented the ætiological agent of the disease. Haagen and Kodama (1934-35) on the contrary suggested that vaccinia virus multiplies diffusely throughout the cytoplasm of the cell and is independent of the formation of the Guarnieri body. Inclusion bodies within the cytoplasm of epithelial cells have also been described by Schütz (1925-26) and by Goodpasture *et al.*, who succeeded in cultivating vaccinia virus in the chorio-allantoic membrane of the developing chick embryo and further demonstrated the presence of inclusion bodies within epithelial and fibroblastic cells. Bland and Canti (1935) demonstrated the presence of inclusion bodies within the fibroblasts of the developing chick embryo during the growth of psittacosis virus in tissue cultivation experiments, and Marchal (1930) in the connective tissue cells and endothelial cells in the lesions of ectromelia in mice. The nature of the general tissue response to vaccinial infection has been extensively investigated by Ledingham (1924), who concluded from his studies that the reaction following vaccination is primarily reticulo-endothelial in character. Ledingham further states that some of the larger inclusion bodies may be nucleolar extrusions.

During the course of histological observations on vaccinial keratitis the authors have repeatedly observed the appearance of acidophilic granules in situations other than those hitherto described. The present investigation has been carried out with the object of elucidating the precise nature of these granules.

Methods.

A laboratory strain of vaccinia virus originally isolated from a case of alastrim by Professor Tulloch was used. It had been maintained by

frequent intratesticular passage through rabbits. 0·1 c.c. of desiccated testis suspended in saline was active to an end titre of 1 : 1,000,000 dilution. Rabbits 3 to 6 months old were lightly anæsthetised, the right cornea scarified with a sharp needle and 0·1 c.c. of a 1 : 10 broth suspension of vaccinia virus instilled into the conjunctival sac. The animals were killed at varying intervals and the corneæ dissected out. Controls were also examined. Tissue was fixed in Helly's fluid and embedded in paraffin and sections 15-20 μ thick were cut and stained by Ford's modification (1934) of Mann's method.

The time factor in the formation of inclusion bodies.

In nine 3-months-old rabbits so treated a chain of events could be traced from the first to the thirteenth day following vaccination. After 48 hours, typical Guarnieri bodies were found in the cytoplasm of the corneal epithelial cells. These bodies were either homogeneous or granular in composition, the latter appearing to consist of an aggregation of highly refractile granules when viewed by dark-ground illumination (*cf.* figs. 4 and 5). Guarnieri bodies increased in numbers up to the sixth day and measured from 2·5 to 3·3 μ in diameter. After this time the corneal epithelium was extensively denuded, making further study impossible.

Forty-eight hours after inoculation there were also observed small granules within the corneal epithelial cells. They measured from 0·34 to 0·68 μ and bore a resemblance to the granules described by Ungermann and Zuelzer, being diffusely scattered throughout the cytoplasm.

Similar changes were noted in the underlying substantia propria. From the first day onwards, many of the fibroblasts in the most superficial layers were seen to contain acidophilic granules (figs. 2 and 3). With Mann's stain the cytoplasm of the fibroblast appeared light blue in colour and the nucleus deeply basophilic. The granules at first measured 0·42-1·67 μ in diameter. On the fourth day and later larger forms made their appearance, many being of similar dimensions to the true Guarnieri body, *i.e.* 1·67-3·3 μ (figs. 4 and 5). The maximum number of fibroblasts showing inclusions was observed from the fifth day onwards. With the progress of the infection marked proliferation of fibroblasts occurred and inclusion bodies made their appearance in the fibroblasts of the deeper layers. Clumps of precisely similar small granules lying free in the spaces of the substantia propria were also encountered. These increased in size and on the fifth day forms measuring up to 3·3 μ were observed.

Infiltration with polymorphs, exhibiting, by the method of staining used, marked eosinophil granularity, occurred from the first day. At first the cells were sharply circumscribed and of healthy appearance. Degeneration was not noticeable until the fourth day, after which many of the polymorphs had broken down, with liberation of their acidophilic granules. These tended to

obscure the histological features, so that it was impossible to distinguish between granules liberated from polymorphs and the free granules noted in the initial stages of the infection.

Inoculation of the cornea by the posterior route.

To inoculate the posterior surface of the cornea a hypodermic needle, bent at an angle of 60°, was introduced at the limbus, 0·2 c.c. of aqueous humour aspirated with a syringe and the fluid replaced with a similar quantity of a 1 : 10 dilution of vaccinia virus suspended in saline. The posterior surface of the cornea was then thoroughly scarified with the needle. Two rabbits treated in this way were killed after 4 and 7 days respectively.

Sections of the cornea stained by Mann's method showed that the corneal epithelium of both the inoculated and the uninoculated eyes was perfectly normal, with neither Guarnieri bodies, free granules nor ulceration. Some of the fibroblasts showed inclusion bodies and there was slight polymorphonuclear infiltration and some desquamation of the endothelial lining of Descemet's membrane.

In general the changes in the substantia propria resembled those seen after inoculation by the superficial route, but they were present in lesser degree.

It was found possible to repeat all the foregoing experiments with a separate strain of vaccinia virus, a commercial preparation of glycerinated calf lymph being employed.

Examination for elementary bodies.

A piece of infected cornea was teased out over a clean slide which was then placed in distilled water for one minute, dried in air, treated with methylated alcohol for 3 minutes, dried in air, treated with Löffler's flagellar mordant for 5 minutes (heating gently for 1 minute), washed with distilled water and then stained with filtered 5 per cent. carbol fuchsin (heating for 1 minute).

Each slide was examined by direct light for elementary bodies and by dark-ground illumination for refractile granules. The results showed that scanty elementary bodies were present after 48 hours and were most numerous from the fifth day onwards. It should be noted that the maximum number of elementary bodies in films coincided with the time at which the greatest number of acidophilic inclusion bodies were observed in fibroblasts.

Control experiments.

In order to investigate the appearances produced in the rabbit cornea by degenerating polymorph leucocytes a series of control experiments was performed on traumatic and bacterial keratitis.

VACCINIAL KERATITIS

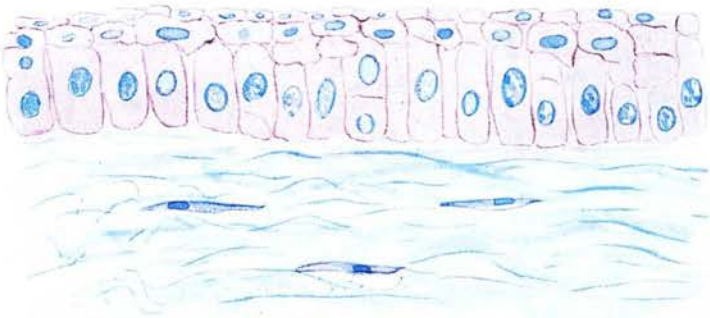


FIG. 1.—Normal rabbit's cornea showing superficial epithelium and substantia propria, with fibroblasts. ×780.

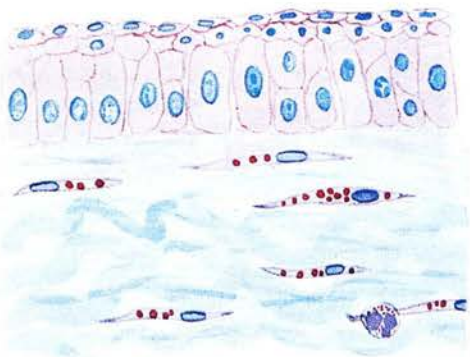


FIG. 2.—Rabbit's cornea one day after inoculation with vaccinia virus, showing acidophilic inclusions in fibroblasts, mainly of small size. ×606.

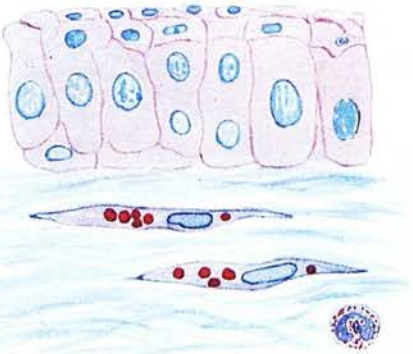


FIG. 3.—Higher magnification of same section: note absence of inclusions in corneal epithelium: a single polymorph is seen. ×910.

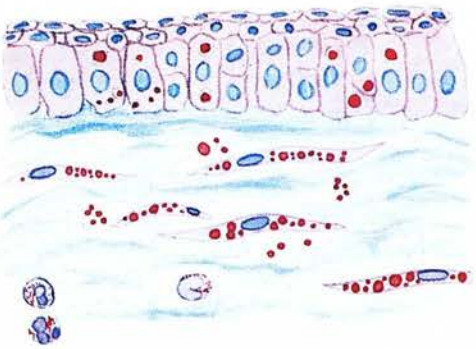


FIG. 4.—Rabbit's cornea four days after inoculation, showing Guarnieri bodies of various sizes in the corneal epithelium and acidophilic inclusions, both large and small, in fibroblasts; extracellular granules large and small; a few polymorphs showing commencing degeneration. ×606.

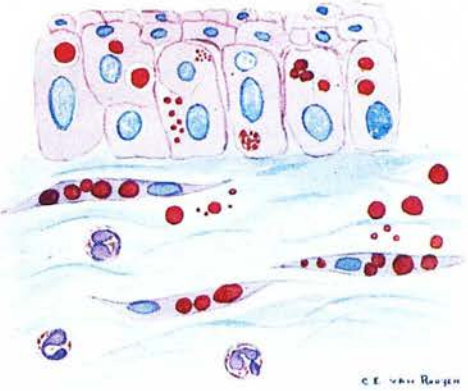


FIG. 5.—Higher magnification of same section. Note presence of Guarnieri bodies, some appearing homogeneous, others granular: a few of the former are surrounded by a characteristic unstained halo. Note the presence of similarly staining bodies in the cytoplasm of fibroblasts. ×910.

All sections stained by Mann's method.

Appearance of the normal cornea. The corneæ of 25 normal rabbits varying in age from 2 to 4 months were cut and stained by Ford's modification of Mann's method. No acidophilic granules, inclusion bodies nor particles, large or small, were seen in either epithelium or substantia propria, nor were eosinophil polymorphs present (fig. 1).

Effect of mechanical trauma. The left corneæ of two rabbits were vigorously scarified with a hypodermic needle point and 0·5 c.c. of broth (similar to that used for the suspension of the vaccinia virus in the experiments already described) instilled into the conjunctival sac. The first animal was killed after 3 days, the second after 5 days. No microscopic differences could be detected between the inoculated and the uninoculated eyes, and it was concluded that the trauma of scarification prior to inoculation did not produce any visible cellular reaction in the corneal tissues.

Effect of chemical irritation. An endeavour was made to produce inflammation of the cornea by means of chemicals. Two rabbits were anæsthetised and one eye of each vigorously scarified with a needle and next painted with turpentine. After 3 days no visible differences could be detected between the eye treated with turpentine and the other. The application was accordingly renewed but no lesions resulted. Sections made 4 and 7 days after the re-application failed to show granules, inclusion bodies or polymorphs.

Pneumococcal keratitis. A strain of type I pneumococcus virulent for mice was used and an acute keratitis was successfully produced in two rabbits by the fourth day after inoculation. Sections obtained 4 and 7 days after inoculation were stained as before. They showed that eosinophilic polymorphic infiltration of the cornea is present on the fourth day and still more notably on the seventh, when many of the cells show degeneration with liberation of their acidophilic granules. Such particles usually did not exceed 1·6 μ in diameter and were approximately of the same size as the acidophilic granules occurring within unruptured polymorphs present in the cornea, circulating blood and bone marrow of the same animal when stained by identical methods. It should be recalled that the largest of the granules in vaccinial infection measured 3·3 μ and were present from the fourth day onwards. No such large extracellular bodies are found in pneumococcal keratitis.

Vaccinial keratitis induced in rabbits previously injected with benzole.

Five rabbits were repeatedly injected with large doses of crude benzole, but only in one case was it found possible to depress the leucocyte count significantly. This rabbit, of 4 lb. weight, was injected subcutaneously with daily doses of 4 c.c. of olive oil-

benzole emulsion for 3 days. The right cornea was then inoculated as before, after which 2 c.c. of olive oil-benzole emulsion were given on the fourth day and 4 c.c. on the ninth day, following which death occurred. The white cell count on the fourth day was 1250 per c.mm., and on the ninth day, immediately prior to death, 625 per c.mm., of which 50 per cent. were polymorphonuclear cells. No histological differences were demonstrable: the number of eosinophil cells present in the sections appeared to be the same, notwithstanding the comparative leucopænia existing in the peripheral circulation.

Attempted agglutination tests.

Eight vaccinated corneæ were removed on the seventh day after infection, a fragment of each retained for histological section and the remainder minced with scissors, ground in a mortar in 5 c.c. of saline, centrifuged at 1000 *r.p.m.* for 30 minutes and the deposited tissue retained. The supernatant fluid was next centrifuged at 14,500 to 15,500 *r.p.m.* for 45 minutes and a part of the deposit spread over a thin slide, the rest being fixed in Helly's solution and embedded in paraffin. The deposit obtained after the first centrifugation was similarly treated.

Histologically the sections of the 8 corneæ stained by Mann's method showed numerous inclusion bodies, but no evidence of inclusions could be found in either stained films or sections made from the first or second centrifuged deposits. On the other hand examination of the same specimens by dark-ground illumination showed the presence of countless granules closely resembling the elementary bodies of vaccinia in shape, size and degree of refractility to light. Films of centrifuged deposit stained by Paschen's method also showed large numbers of elementary bodies.

From these results it was concluded that the acidophilic inclusion bodies were readily broken up by the procedure described, and thus any attempt to apply an agglutination test was abandoned.

Use of rabbits previously immunised against vaccinia virus.

Three rabbits which had been repeatedly inoculated with graded doses of live vaccinia virus for a period of over 8 weeks were subjected to test. As before, the right eye of each animal was thoroughly scarified with a needle and 0·5 c.c. of a 1 : 10 saline suspension of desiccated vaccinia testis placed in the conjunctival sac. Simultaneously two normal animals were similarly treated, to prove the pathogenicity of the specimen of virus used. The test animals were killed after 6, 7 and 10 days. Both control animals showed gross evidence of a severe keratitis before the sixth day and microscopically exhibited the typical changes of vaccinial

infection. The immune animals, however, showed no evidence of keratitis either naked-eye or microscopically: only a few scanty extracellular granules were to be seen in the preparations examined, neither Guarnieri bodies nor fibroblastic inclusion bodies being present. These results confirm the work of Sobernheim (1925).

Discussion.

Following vaccination of the rabbit's cornea a characteristic series of pathological changes is induced, the main interest of which centres on the presence of acidophilic inclusions in the fibroblasts of the substantia propria. We have failed to reproduce these inclusions in other varieties of keratitis, whether of mechanical, chemical or bacterial origin.

The question whether they were acidophilic granules which had been extruded from degenerate polymorph leucocytes was considered, but parallel observations on pneumococcal keratitis showed that although eosinophilic infiltration and degeneration were present in this type of infection, there were no inclusion bodies in the fibroblasts. Also the inclusion bodies were noted as early as 24 hours after vaccination of the cornea, long before polymorphic degeneration had commenced.

It is suggested therefore that these structures represent a phase in the growth of vaccinia virus in the rabbit's cornea, the more so in that they fail to appear in rabbits immunised against the disease. Indeed, it is probable that they are colonies of the virus itself, for they have been shown to increase from 0·42 to 3·3 μ in diameter from the first to the fifth day following inoculation. Critical microscopic scrutiny of these structures by direct and oblique illumination has revealed that they are granular in composition and closely resemble a mass of vaccinial elementary bodies. This hypothesis is supported by data which show that the period of infection during which the maximum number of inclusion bodies is encountered, invariably coincides with the time at which the greatest number of elementary bodies are observed in the same tissue. In conclusion, an experiment has been described in which it was shown that these inclusions were delicate structures that depended for the cohesion of their constituents on the integrity of the cell containing them.

Conclusions.

1. Some cytological features of vaccinial keratitis in the rabbit are described in detail from the first to the thirteenth day following vaccination.

2. The presence of acidophilic inclusion bodies within fibroblasts is described. These appear to be a specific feature of vaccinial

keratitis and have not been observed in keratitis produced by bacterial, chemical or mechanical means.

3. The significance of these structures has been investigated by appropriate methods and arguments in favour of their being aggregates of elementary bodies, which constitute the virus itself, are adduced.

4. It has been demonstrated that both Guarnieri bodies and the fibroblastic inclusions fail to develop in the corneæ of rabbits which have been previously actively immunised against vaccinia virus.

The authors desire to express their indebtedness to Professor Mackie for much helpful guidance and constructive criticism. They also wish to thank Professor Cappell, Dr Mervyn Gordon and Professor Tulloch for the interest they have shown in these investigations. One of us (C. E. v. R.) would like to acknowledge his indebtedness to the late Professor Paschen of Hamburg for instruction in the staining and recognition of elementary bodies.

During the course of this work A. J. R. has held a Crichton Research Scholarship, and C. E. v. R. an expenses grant from the Medical Research Council.

REFERENCES.

BLAND, J. O. W., AND CANTI, R. G. 1935. this *Journal*, xl. 231.

FORD, J. . . . . 1934. *J. Roy. Micr. Soc.*, series 3, liv. 263.

GOODPASTURE, E. W., WOODRUFF, A. M., AND BUDDINGH, G. J. 1932. *Amer. J. Path.*, viii. 271.

GUARNIERI, G. . . . . 1892. *Arch. Sci. Med.*, xvi. 403.

HAAGEN, E., AND KODAMA, M. 1934-35. *Zbl. Bakt.*, Abt. I. Orig., cxxxiii. 23.

LEDINGHAM, J. C. G. . . . 1924. *Brit. J. Exp. Path.*, v. 332.

MARCHAL, J. . . . . 1930. this *Journal*, xxxiii. 713.

PAUL, G. . . . . 1914-15. *Cbl. Bakt.*, Abt. I. Orig., lxxv. 518.

„ . . . . . 1917-18. *Ibid.*, Abt. I. Orig., lxxx. 361.

VON PROWAZEK, S., AND MIYAJI, S. 1914-15. *Zbl. Bakt.*, Abt. I. Orig., lxxv. 144.

SCHÜTZ, V. . . . . 1925-26. *Z. Hyg.*, cv. 1.

SOBERNHEIM, G. . . . . 1925. *Ergebn. Hyg. Bakt.*, vii. 133.

UNGERMANN, E., AND ZUELZER, M. 1920. *Arb. Reichsgesundhamt.*, lii. 41.

PRINTED IN GREAT BRITAIN BY OLIVER AND BOYD LTD., EDINBURGH

Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY
Vol. XLIX., No. 2, pp. 345-349, 1939.

The Chemical Composition of the Molluscum Contagiosum Inclusion Body

BY

C. E. VAN ROOYEN

From the Department of Bacteriology, Edinburgh University

616—006 . 393 : 577 . 1

THE CHEMICAL COMPOSITION OF THE MOLLUSCUM CONTAGIOSUM INCLUSION BODY.

C. E. VAN ROOYEN.

From the Department of Bacteriology, Edinburgh University.

(PLATES LXII.-LXIV.)

IN a previous publication the structure of the molluscum inclusion body was described on the basis of micromanipulation and micro-dissection studies (van Rooyen, 1938). Among the various observations recorded it was shown that the body was composed of an outer covering or membrane of varying thickness, inside which were numerous elementary bodies embedded in a gelatinous matrix. The writer has now extended these investigations and has succeeded in obtaining some information regarding the chemical character of the outer envelope, as well as the relative distribution of mineral matter in the different components after micro-incineration.

Tests for glycogen.

Following the work of Rice (1936) and Thygeson (1938), who showed that the inclusion bodies of trachoma gave the iodine reaction for glycogen, the writer has applied the same tests to the molluscum body, and found that it reacts in a similar manner. The results were as follows.

Reaction to iodine. Films fixed by gently heating or with alcohol, were treated with undiluted Lugol's iodine solution for one minute, washed with distilled water and examined under the microscope. The inclusions assumed a dark brown colour identical with that of liver cells after similar treatment.

Effect of heat. The brown colour so produced could be made to disappear by gently warming the slide and it reappeared on cooling. A film of liver tissue similarly treated behaved in a similar way; this suggests that the molluscum body contains a substance which is in all probability glycogen or some similar carbohydrate.

The action of saliva. If a film of suitable tissue is fixed by heat and treated with fresh human saliva for 24 hours the iodine-staining

substance disappears. A control slide exposed for 24 hours to saliva which has been previously boiled for one hour shows the same appearances as an untreated slide.

This strongly suggests that the reacting material is carbohydrate in nature since it can be removed by exposing it to the action of the enzymes, amylase and ptyalin, of saliva.

Location of the carbohydrate substance.

Experiments were next made to see if any of the individual components of the molluscum body possessed a special affinity for iodine. With the aid of a micro-manipulating microscope a fresh smear of infected tissues was stained *intra vitam* by mixing it with a weak solution of iodine in saline and placed over a dissecting chamber (van Rooyen). A single inclusion was isolated by the method originally devised by Schouten (1936) for the isolation of single bacterial cells, using a glass micromanipulating needle with a loop about 20-25 μ in diameter at its extremity, to snare and pick up any inclusion body selected (figs. 1 and 2). The structure was then dissected with sharp needles as previously described (van Rooyen) and it was found that only the outer covering or envelope exhibited an affinity for iodine ; neither the elementary bodies nor the jelly-like matrix in which they are embedded seemed to take up the brown colour. The carbohydrate substance is thus apparently limited to the outer covering of the inclusion and is absent from its contents. I also carried out the test recommended by Pringsheim and Krüger (1932) for the microscopic detection of glycogen but it gave only a feebly positive response. The test for cellulose employed by Ashworth (1922) was also attempted, but was negative.

Micro-incineration studies.

Molluscum lesions were excised from patients, fixed in a mixture of 90 c.c. of 3 per cent. potassium dichromate and 10 c.c. of concentrated neutral formalin (Gage, 1936), and embedded in paraffin. Serial sections 4-7 μ in thickness were floated on absolute alcohol and mounted on special heat-resisting glass or quartz slides. Each alternate strip of sections was stained by Mann's method for inclusion bodies and the remainder were placed in an electric furnace and incinerated without preliminary removal of the paraffin. With sections prepared from tissue fixed in Zenker or Helly's fixative the paraffin was first removed with xylol and alcohol. The section was treated with iodine solution and repeated washing in water to remove all traces of mercuric salts and then thoroughly dehydrated with alcohol and carefully dried prior to heating.

The method of heating eventually adopted after a number of trial experiments was as follows. Each slide was placed in the cold electric furnace and the current switched on for a period of 35 minutes, at the end of which the slide had usually reached a dull red heat. The oven was allowed to cool and the slide removed. The section usually presented a greyish white colour to the naked eye. It is not possible to state the precise temperature

CHEMISTRY OF MOLLUSCUM INCLUSION BODY

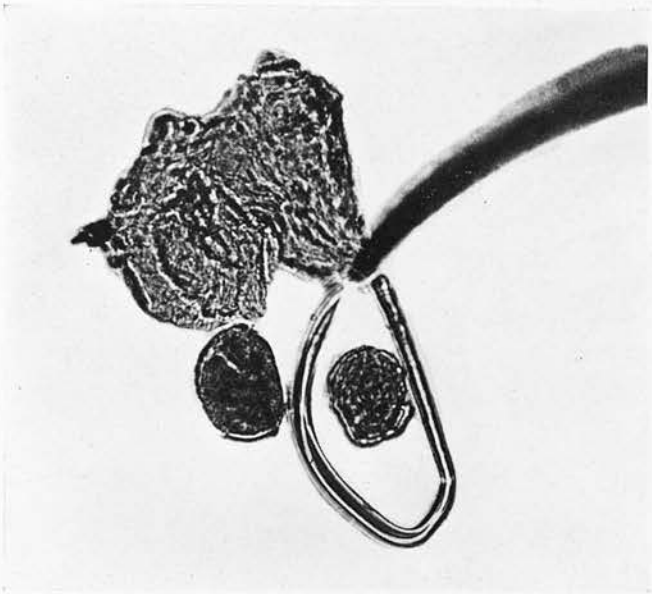


FIG. 1.

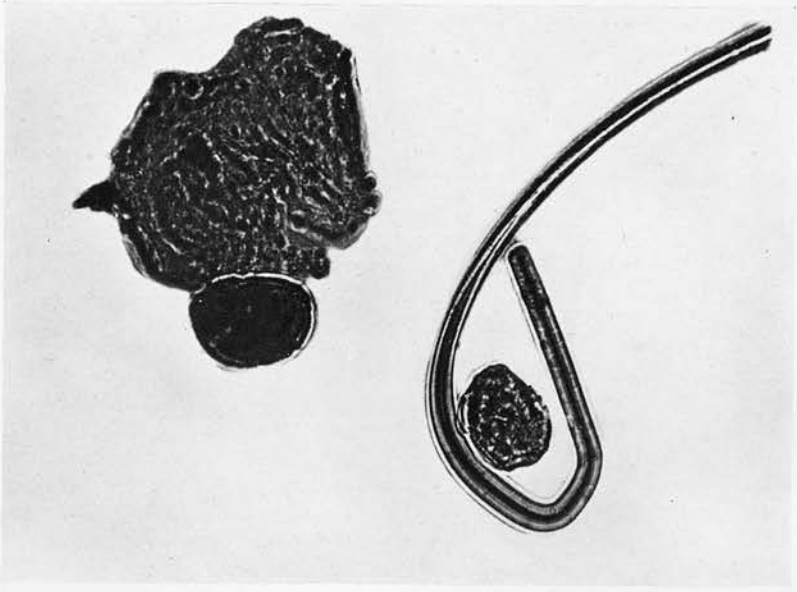


FIG. 2.

FIGS. 1 and 2.—Method of removing a single molluscum body with the micro-manipulating loop. Fresh preparation stained with Lugol's iodine. ×600.

CHEMISTRY OF MOLLUSCUM INCLUSION BODY

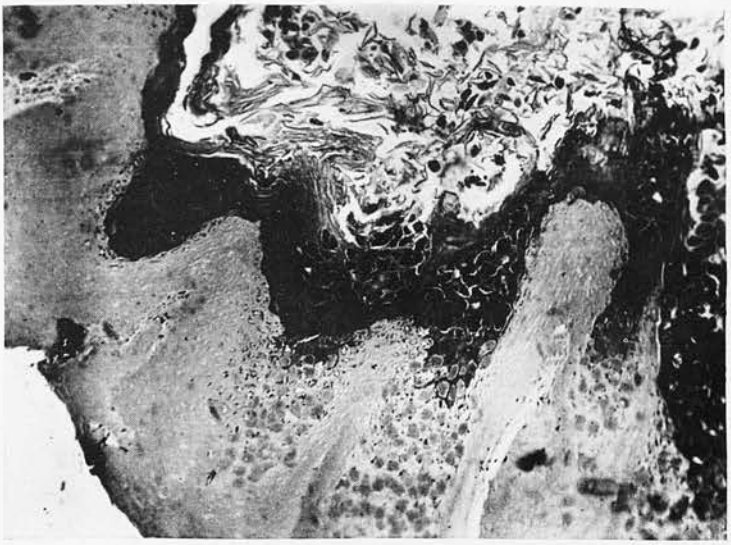


FIG. 3.—Section of molluscum tissue. The large inclusions appear black. Mann's stain. ×100.

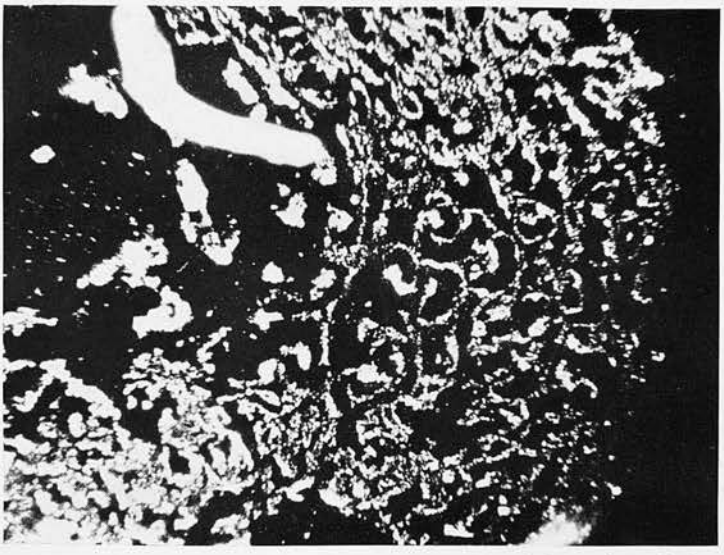


FIG. 4.—Section similar to that shown in fig. 1 after micro-incineration; dark ground illumination. Note the white residue of inorganic salts indicating the outline of individual epithelial cells and within these the ashed remains of inclusion bodies. ×160.

CHEMISTRY OF MOLLUSCUM INCLUSION BODY

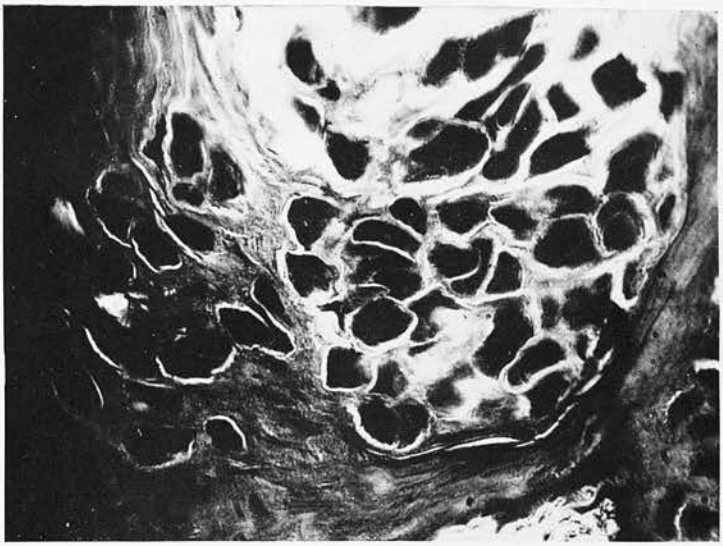


FIG. 5.—High power view of molluscum bodies showing their comparatively homogeneous and uniformly staining character. The inclusions fill the entire cell. Mann's stain. ×330.

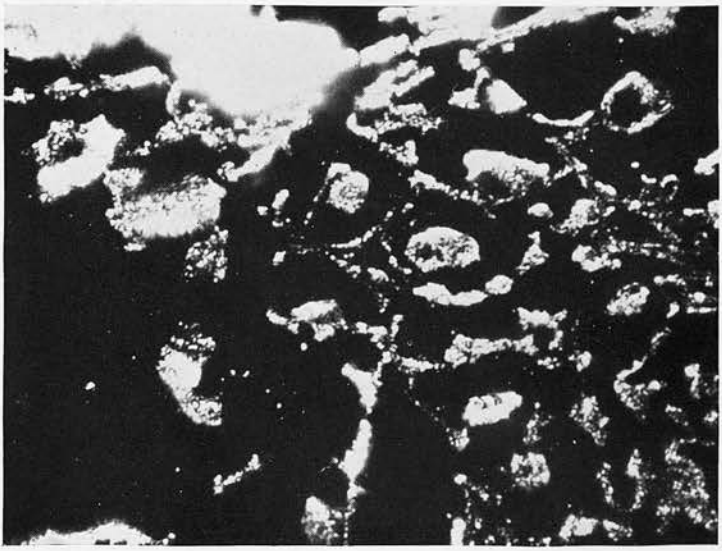


FIG. 6.—High power view of incinerated molluscum bodies showing the outline of the separate epithelial cells and the granular composition of the inclusion bodies. Oblique illumination. ×500.

reached at the conclusion of 30 minutes' heating, but according to the maker's instructions the furnace was claimed to reach 1000° C. in 45 minutes, and it is assumed that the temperature employed in these experiments was 600° C. at least. For accurate information on the amount of heat required the reader is referred to the writings of Gage, who has recommended the following successive exposures: 10 minutes at 100°, 5 minutes at 150°, 5 minutes at 206°, 5 minutes at 272°, 5 minutes at 354°, 5 minutes at 458° and 1 minute at 600° C.

Two microscopes were used, one fitted with a dark-ground condenser, the other with an ordinary Abbé condenser; a comparison microscope if available is ideal for the purpose. The first set of stained serial sections was focussed under direct light and the immediately succeeding group of incinerated sections under the dark-field instrument. It was thus possible to compare the appearances of the stained and the incinerated inclusion bodies under both low and high powers.

The results are illustrated in figs. 3-6. Sections of a molluscum lesion stained by Mann's method (figs. 3 and 5) exhibit the typical acidophilic molluscum bodies within the cytoplasm of infected epithelial cells; they appear dark in the photographs.

In the incinerated sections (figs. 4 and 6) the intercellular substance has almost entirely disappeared and only the faint outlines of individual epithelial cells remain visible, whereas the contents can be distinguished as clumps and masses of glistening white material within the cells.

From a comparison of successive serial sections under the high power it will be noticed that the stained molluscum body (fig. 5) appears as a homogeneous, uniformly staining structure, whereas after incineration it consists of a collection of white granular material which is highly refractile (fig. 6). This white residue which resists heating up to approximately 600° C. is in all probability calcium carbonate or oxide,* first on account of its white colour, secondly because when a drop of dilute acetic or hydrochloric acid was placed on the preparation and watched under the microscope the material seemed to effervesce, small bubbles of gas developing, and thirdly, when the residue was taken up on a platinum loop, moistened with concentrated HCl and heated in the bunsen flame a brick red colour developed in the flame, suggesting the presence of calcium. As to the source of this material, the large masses of calcium compound inside the cells were obviously the incinerated remains of inclusion bodies or their matrix, but it was difficult to tell whether this was mainly derived from the outer envelope, from the elementary bodies and their matrix within, or from both these sites.

With a view to obtaining further information on this point, a number of stained and incinerated sections were carefully examined by dark-ground illumination under a magnification of 1000. The results showed that whereas the outline of the stained inclusions was regular in shape, after incineration they assumed

* Calcium carbonate is converted to calcium oxide *c.* 800° C.

an irregular outline and seemed to be composed of granular material. These appearances suggested that the envelope of the molluscum body had disappeared completely after heating and that the calcium residue was largely if not entirely composed of the ashed contents. However, in order to check this deduction and to be certain of the appearance of incinerated elementary bodies, a smear preparation was made from fresh tissues known to contain numerous elementary bodies, and after heating it was found that they too exhibited precisely similar clumps of material in their interior.

Discussion.

The facts recorded in this article regarding the chemical reactions of the molluscum body following its treatment with iodine are similar to observations already reported by Rice and by Thygeson concerning the Prowazek-Halberstaedter inclusions of trachoma, and suggest that the outer coverings of these two types of inclusion body are composed mainly of a carbohydrate substance. It should be mentioned however that the molluscum body exhibits a marked avidity for iodine and that it seemed to be less easy to remove the carbohydrate-reacting properties of the molluscum body by digestion with saliva than was found to be the case by Rice and Thygeson for trachoma bodies.

The results obtained after micro-incineration are also in agreement with the hypothesis that the outer envelope of the molluscum body is largely composed of carbohydrate, since after heating to 600° C. no trace of the outer coat could be detected.

It is apparent that the residue remaining is derived from the elementary bodies and their matrix within the inclusion, and furthermore that these are rich in mineral matter which is in all probability calcium. A similar observation has been made in connection with the inclusion body of fowl-pox by Danks (1932), who found that, after heating, the inclusion bodies appeared as aggregations of greyish white particles of ash. These, he believed, represented the remains of elementary bodies.

Conclusions.

1. The chemical reactions of the outer covering of the molluscum contagiosum inclusion body have been investigated.

2. Tests with Lugol's iodine indicate that this envelope is mainly composed of carbohydrate which can be largely removed by preliminary digestion with fresh human saliva but not by boiled saliva.

3. After micro-incineration the outer covering of the molluscum body is burnt off completely.

4. The mineral residue of the incinerated inclusion is probably mainly derived from the elementary bodies but some may possibly come from the matrix in which they are embedded.

The writer desires to acknowledge his indebtedness to Dr C. G. Anderson and Dr S. W. Challinor for many helpful suggestions received during the course of the investigations; also to Prof. Mackie for much constructive criticism. The work was performed with the aid of an expenses grant from the Medical Research Council.

REFERENCES.

ASHWORTH, J. H.	1922-25.	*Trans. Roy. Soc. Edinb.*, liii. 301.
DANKS, W. B. C.	1932.	*Amer. J. Path.*, viii. 711.
GAGE, S. H.	1936.	The microscope, 16th ed., *New York*, p. 521.
PRINGSHEIM, H., AND KRÜGER, D.	1932.	Handbuch der Pflanzenanalyse, *Vienna*, vol. ii., p. 874.
RICE, C. E.	1936.	*Amer. J. Ophth.*, xix. 1.
VAN ROOYEN, C. E. . . .	1938.	This *Journal*, xlvi. 425.
SCHOUTEN, S. L.	1936.	De Micromanipulator, *Amsterdam*, pp. 225 and 297 *et seq.*
THYGESON, P.	1938.	*Amer. J. Path.*, xiv. 455.

PRINTED IN GREAT BRITAIN BY OLIVER AND BOYD LTD., EDINBURGH

HEINBECKER, P., and O'LEARY, J. L. (1930), *Anat. Rec.*, **45**, 219.
HIMMELWEIT, F. (1938), *Brit. J. exp. Path.*, **19**, 108.
HINDLE, E. (1932), *Nature*, **129**, 796.
HINDLE, E., and STEVENSON, A. C. (1929–30), *Trans R. Soc. trop. Med. Hyg.*, **23**, 327.
HORNING, E. S., and FINDLAY, G. M. (1934), *J. R. micr. Soc.*, **54**, 9.
ISHIMITSU, K. (1937), *Jap. J. exp. Med.*, **15**, 185.
KUTTNER, A. G., and WANG, S-H. (1934), *J. exp. Med.*, **60**, 773.
LEE, J. (1933–4), *Proc. Soc. exp. Biol., N.Y.*, **31**, 383.
—— (1936), *Amer. J. Path.*, **12**, 217.
LIPSCHÜTZ, B. (1921), *Arch. Derm. Syph., Wien*, **136**, 428.
LUCAS, A. M., and HERRMANN, W. W. (1935), *Amer. J. Path.*, **11**, 969.
LUDFORD, R. J. (1930), *A System of Bacteriology*, vol. **7**, H.M. Stationery Office, London.
MCCORDOCK, H. A., and SMITH, M. G. (1934), *Amer. J. Dis. Child.*, **47**, 771.
NEGRI-LUZZANI, L. (1905), *Z. Hyg. InfektKr.*, **49**, 305.
—— (1913), *Ann. Inst. Pasteur*, **27**, 907, 1039.
NICOLAU, S., and KOPCIOWSKA, L. (1936), *Ann. Inst. Pasteur*, **57**, 244.
NICOLAU, S., KOPCIOWSKA, L., and BALMUS, G. (1933), *C.R. Soc. Biol., Paris*, **113**, 851.
OLITSKY, P. K., and HARFORD, C. G. (1937), *Amer. J. Path.*, **13**, 729.
PAPPENHEIMER, A. M., and HAWTHORNE, J. J. (1936), *Amer. J. Path.*, **12**, 625.
PAPPENHEIMER, A. M., and MAECHLING, E. H. (1934), *Amer. J. Path.*, **10**, 577.
RECTOR, L. E., and RECTOR, E. J. (1933), *Amer. J. Path.*, **9**, 587.
—— —— (1934), *Amer. J. Path.*, **10**, 629.
RICH, A. R. (1932), *Johns Hopk. Hosp. Bull.*, **51**, 346.
RIVERS, T. M. (1928), *Amer. J. Path.*, **4**, 91.
RUSSELL, D. S. (1932), *J. Path. Bact.*, **35**, 625.
SCOTT, G. H. (1930), *C.R. Acad. Sci., Paris*, **190**, 1073, 1323.
SHORTT, H. E., and LAHIRI, B. N. (1934), *Indian J. med. Res.*, **21**, 587.
SLANETZ, C. A., and SMETANA, H. (1937), *J. exp. Med.*, **66**, 653.
SMITH, J. H. (1930), *A System of Bacteriology*, vol. **7**, H.M. Stationery Office, London.
SPOONER, E. T. C. (1938), *J. Hyg., Camb.*, **38**, 79.
ST. JOHN-BROOKS, R. (1930), *A System of Bacteriology*, vol. **7**, H.M. Stationery Office, London.
VAN ROOYEN, C. E. (1938), *J. Path. Bact.*, **46**, 425.
VONGLAHN, W. C., and PAPPENHEIMER, A. M. (1925), *Amer. J. Path.*, **1**, 445.
VON PROWAZEK, S. (1907), *Arch. Protistenk.*, **10**, 336.
WOLF, A., and ORTON, S. T. (1932), *Bull. neurol. Inst. N.Y.*, **2**, 194.
WOODRUFF, C. E., and GOODPASTURE, E. W. (1929), *Amer. J. Path.*, **5**, 1.

CHAPTER VIII

SEROLOGICAL TECHNIQUE IN THE STUDY OF HUMAN VIRUS DISEASES

RECENT progress has shown that the immunological characteristics of the filterable viruses are analogous to those of the visible bacteria, and many serological phenomena—agglutination, precipitation, complement-fixation and neutralization tests—as applied to bacteria have been demonstrated with viruses. Minor modifications of existing technique as used with bacteria are, however, necessary to render such tests suitable for the study of virus agents. The principal difficulty, in fact, involves preparation of suitable antigens. This is due to the fact that, while bacteria are readily cultivable on dead media, viruses can be grown only in presence of living cells. Therefore, for certain serological tests it may be necessary to separate the virus agents from the tissues containing them. This may be done by differential centrifugalisation and repeated washing of tissue extracts, so that suspensions of elementary bodies are prepared. Although relatively pure suspensions of elementary bodies are thus obtainable it is impossible to eliminate all traces of protein derived from the parasitized cells. It is therefore necessary to include a special control in the reactions; this consists of an extract of normal tissue which has been prepared from the corresponding organ of the same species as that of the animal infected with the virus under investigation. This extract is prepared exactly as is that of the infected material.

For example, if an elementary body suspension obtained from rabbit's testicle is to be agglutinated with antivaccinial serum, then the antigen control should consist of a saline extract of normal rabbit's testis, which has been subjected to the same treatment as the infected tissue.

The serum controls demand special attention and sometimes two are required for each serum tested. For example, if an antivaccinial serum has been prepared by immunizing a rabbit with vaccinia virus cultivated on the chorio-allantoic membrane of the chick embryo, then it should be controlled with one sample of serum derived from another rabbit after immunization with normal chick embryo and a second from a normal rabbit. In dealing with homologous tissue, e.g. when a rabbit is actively immunized with vaccinia virus cultivated in rabbit testis, a single control consisting of normal rabbit serum suffices.

THE ACTIVE IMMUNIZATION OF ANIMALS AGAINST VIRUSES, AND THE PREPARATION OF ANTISERA

Vaccinia

Rabbits can be immunized with comparative ease against vaccinia virus. A normal animal is inoculated subcutaneously with 0·25 c.c. of a 1:1,000 dilution of vaccinia virus contained in desiccated testis. After 3 to 5 days a cutaneous reaction develops at the site of injection, and thereafter the condition may either generalize (see Douglas, Smith, and Price, 1929), or else the lesion may remain localized and subsequently retrogress, depending on the virulence of the strain of virus employed. Recovered rabbits are resistant to reinfection, and their immunity may be exalted by repeated doses of virus which may be administered either intravenously

PART III.

SEROLOGICAL TECHNIQUE IN THE STUDY OF VIRUS INFECTIONS.

Like bacterial diseases virus infections are accompanied by the formation of specific antibodies. Two special studies were made on the sera of rabbits infected with Shope fibroma and rabbit myxoma virus and the presence of complement-fixing antibodies was found in each of these conditions, thus bringing them into line with the behaviour of other virus infections described by earlier investigators. I have also inquired into the relationship of virus neutralizing antibodies to complement-fixing antibodies in Shope fibroma infection.

In view of the discovery of Dedrick (who was able to transform Shope fibroma into myxoma virus) I carried out experiments to test the degree of specificity of such virus antibodies, but cross complement-fixation tests showed that no immunological interrelationship could be found to exist between these two viruses. The remainder of the text deals with a critical review of the literature with special emphasis on the accurate interpretation of results and the importance of proper control tests.

or intraperitoneally (see Gordon, 1925; Craigie and Tulloch, 1931; Craigie and Wishart, 1934*a*; Smith, 1930).

Hyperimmune sera prepared in this manner contain agglutinins, precipitins, complement-fixing and virus-neutralizing antibodies, which are usually demonstrable about 14 days after the date of infection (see Craigie and Wishart, 1934*a*). There is no standard method for estimating accurately the potency of such sera, but about 1 c.c. of an active antivaccinial serum should protect against 1,000 minimal infecting skin doses, according to Andrewes (1928). The activity of the immune bodies in rabbit's serum has been shown to reside in the globulin fraction (Findlay, 1931).

Horses. Ledingham, Morgan, and Petrie (1931) actively immunized a horse against vaccinia virus, with the aim of studying the properties of such serum as well as providing themselves with antivaccinial serum for use in cases of post-vaccinal encephalitis or smallpox.

The animal was first immunized with formolized diphtheria toxin and tetanus toxin of which 5 doses were given. Subsequently it was injected repeatedly with glycerolated calf lymph (government strain), and during this period it was given a routine dose of tetanus toxoid every 2 or 3 months. The dosage of virus consisted of an initial subcutaneous innoculation of a 1:10 dilution of lymph, which was scarified into an area of shaved skin about a foot square, situated on one side of the horse's neck. Fifty hours later, a crop of about 200 vesicles developed, then gradually subsided and healed, so that after 35 days the skin had returned to its normal condition. The animal was now hyperimmunized by a further inoculation of 10 c.c. of lymph given subcutaneously. This was followed 8 weeks later by 71 c.c. intravenously, a month afterwards by 40 c.c. intravenously, and finally with 130 c.c. given intramuscularly. The total bulk of glycerolated calf lymph introduced was 282·5 c.c. administered over a period of 9 months.

Sample bleedings, varying in amount from 0·75 to 2·2 litres, were made at different periods during the course of immunization, and the last quantity of blood was withdrawn about 3½ months after the final intramuscular dose.

From their researches, Ledingham *et al.* found that the euglobulin and pseudo-globulin fractions of the serum contained the greatest amount of antiviral body. The euglobulin possessed the greatest concentration of antibody per gramme of protein, but its absolute amount fell below that present in the pseudo-globulin fraction. Such hyperimmune horse antivaccinial sera, when administered subcutaneously with vaccinia virus of either testicular or dermal origin, proved highly effective in controlling experimental infections in rabbits.

Herpes

Guinea-pigs. Antivaccinial or antiherpetic immune serum may be prepared by injecting these animals repeatedly with large doses of live virus.

Bedson and Bland (1929) recommend that they be given 6 to 8 doses of 1·0 to 2·0 c.c. of a 10 per cent. suspension of virus material, administered intraperitoneally, and sera prepared thus were shown to contain complement-fixing antibodies for these two viruses.

Psittacosis

Guinea-pigs are comparatively resistant to psittacosis virus and Bedson (1933) has successfully immunized them against this agent. A mouse was first inoculated with virus, its spleen removed and extracted with saline

to make a 10 per cent. suspension of tissue. Guinea-pigs received 2 to 3 c.c. of the 10 per cent. extract administered intraperitoneally at weekly intervals, with periodic rests of 1 to 2 months; each of these animals received from 10 to 15 injections, and their sera contained both virus-neutralizing and complement-fixing antibodies.

Poliomyelitis

The horse has been immunized with a strain of poliomyelitis virus by Fairbrother (1930), who used highly infective tissue derived from the spinal cord and Rolandic area of monkeys which had become paralysed following intracerebral injection with the virus (see also Fairbrother and Hurst, 1930). Five different dilutions of cord-brain suspension were prepared, namely, 0·5 per cent., 1 per cent., 5 per cent., 10 per cent., and 20 per cent.; these were left overnight at +4° C., filtered through a Berkefeld N candle, and then injected into the horse. The animal received at first 50 c.c. of a 0·5 per cent. suspension administered in 3 equal doses at intervals of 3 days, then 75 c.c. of a 1 per cent. extract in 2 doses at 4-day intervals, then 100 c.c. of a 5 per cent. concentration distributed over 4 inoculations at 3-day intervals, then 150 c.c. of a 10 per cent. solution at 3-day intervals, and finally, 200 c.c. of a 20 per cent. strength was introduced in 3 doses spaced 3 and 4 days apart.

The horse thus received 15 separate inoculations over a space of 2 months. Each injection was given at 3- to 4-day intervals and a period of 7 days was allowed to lapse between each ascending series of dilutions. During the earlier part of the course of immunization, the horse was given a few small doses of tetanus toxoid as a precautionary measure.

Poliomyelitis antisera obtained in this manner were tested for *in vitro* neutralizing properties. Increasing amounts of virus, ranging from 0·1 c.c. to 0·5 c.c. of a 5 per cent. suspension of cord, were mixed with 0·4 to 0·8 c.c. of antiserum, incubated at 37° C. for $1\frac{1}{2}$ to $2\frac{1}{2}$ hours, left overnight at 4° C., and 0·5 c.c. of the mixture inoculated intracerebrally in a monkey.

By this method, Fairbrother (1930) demonstrated *in vitro* the presence of virus-neutralizing antibody in the serum of a horse after it had been immunized with poliomyelitis virus. Curative, prophylactic, and other *in vivo* tests on monkeys were also tried, but unfortunately the antiserum failed to modify or prevent the development of the disease in monkeys infected intracerebrally with virus.

Fairbrother rightly draws attention to the fact that the result of this experiment does not necessarily prove that antiserum is useless for the treatment of patients, because the circumstances attending experimental poliomyelitis in the monkey are entirely different from those prevailing in the case of naturally acquired infection in man. For example, the condition in monkeys is virtually 100 per cent. fatal, against 10 to 20 per cent. in man, and moreover in the case of the former, the virus is being implanted directly into traumatized nervous tissue, so that it rapidly enters the nerve cells and establishes itself considerably in advance of the arrival of antibody from the blood.

Influenza

The horse has been shown to be resistant to this agent; influenza virus administered intratracheally in 4 animals yielded negative results (Laidlaw, Smith, Andrewes, and Dunkin, 1935). The animals were hyperimmunized by giving each horse 12 subcutaneous injections of about 15 c.c. in bulk, prepared from infected ferrets' nasal mucosae, over a period of 4 months.

This was followed by 6 further injections of virus contained in ferret lungs, over a space of 8 weeks, at the end of which the horse's serum was found to neutralize influenza virus in a dilution of 1 in 100.

A second horse was immunized with 50 c.c. doses of ferret-lung emulsion for 2 months, the last 3 injections of virus consisting of a mixture of both the human and porcine strains. The animal appeared to exhibit considerable reactions to the latter inoculations.

A third horse was injected with 6 subcutaneous doses of ferret-lung suspension containing Shope's strain of hog influenza virus, the dosage varying from 30 to 50 c.c. over a month. After one month's rest, hyper-immunization was continued and the animal received, without signs of ill-effects, the remaining injections consisting of a mixture of human and porcine strains of virus cultivated in ferrets.

Hyperimmune sera were next subjected to fractionation and concentration, and it was found that the most active part resided in the pseudo-globulin fraction of the serum, which could be precipitated by salting-out with 12 to 16 per cent. sodium sulphate.

Ferrets. Apparently these animals could only be immunized against either human or swine influenza virus, cultivated in ferret-lung tissue, by inoculating them intranasally. Parenteral inoculation by other routes with either living or dead virus failed to confer immunity to infection. Animals successfully immunized retained this property for about 6 months and thereafter became susceptible to a second attack, unless reinforced by a further injection which could be given subcutaneously.

Mice were also immunized against the virus of human or porcine influenza by administering three or more doses of virus subcutaneously (see Smith, Andrewes, and Laidlaw, 1935). Mice have also been immunized against influenza virus by inoculating them with tissue extracts prepared by Hoyle and Fairbrother (1937), according to the technique adopted by Eagles and Ledingham (1932) for the purification of vaccinia virus. A 10 per cent. suspension of infected mouse lung in saline was ground up with sand; centrifuged for 30 minutes at 400 r.p.m.; 20 c.c. of the suspension was centrifuged for 45 minutes at 13,000 r.p.m.; the supernatant fluid was discarded; the deposit was resuspended in 10 c.c. of saline and centrifuged at 4,000 r.p.m. for 20 minutes; the supernatant fluid was withdrawn and centrifuged at 13,000 r.p.m. for 30 minutes; the supernatant fluid was discarded; the deposit was resuspended in 2 c.c. of saline and lightly centrifuged at 4,000 r.p.m. for 20 minutes to throw down large particles, and the supernatant fluid was used as antigen. The final product was found to be slightly opalescent and was about 10 times as infective as the original mouse lung. Under dark-ground illumination, it showed innumerable minute particles of apparently uniform size, and accordingly Hoyle and Fairbrother have regarded such preparations as 'elementary body suspensions', although stained elementary bodies were not demonstrated by them. Such suspensions did not prove of value as antigens for complement-fixation tests, but they were useful for immunizing mice against influenza virus, the animals being given 2 doses intraperitoneally, the first 0·1 c.c., and the second 0·15 c.c., administered 4 days later. Eleven days after the last dose, the animals were tested for susceptibility by inoculating them intranasally with influenza virus and were found to be resistant to infection.

Rift Valley Fever

Mice are so highly susceptible to infection with this agent that difficulty has been experienced in attempting to immunize them with either

phenolized or formolized vaccines of virus. Mackenzie (1935) has successfully overcome the difficulty by employing virus suspensions which have been inactivated by exposing them to the photodynamic action of methylene blue, used previously by Perdrau and Todd (1933) in connexion with their studies on dog distemper. The blood of 3 mice moribund from Rift Valley virus was added to 5 c.c. of physiological saline and centrifuged at 2,000 r.p.m., the supernatant fluid was removed and mixed with an equal volume of 1 : 50,000 dilution of methylene blue. The fluid was then poured into a petri dish and exposed for 30 minutes to the light of a 100 c.p. pointolite lamp. Mice inoculated intraperitoneally with 1 c.c. of this vaccine subsequently proved to be resistant to infection with live virus, when tested for immunity 7 days later.

TESTS FOR VIRUS-NEUTRALIZING ANTIBODIES

Tests for virus-neutralizing antibodies have been employed for three different purposes, namely: (1) to assess the potency of therapeutic antisera with a view to standardizing them; (2) for detecting the presence of virus-neutralizing antibodies in human or animal sera for diagnostic or epidemiological purposes; and lastly, (3) to identify viruses or to investigate their antigenic structure. The general principle on which these tests are based is the same, for in the majority of instances the serum to be tested is mixed with the virus in suitable proportions, incubated for a short time, and inoculated into a susceptible animal. In some of these tests the amount of virus used is kept constant and different dilutions of serum are added, such as in the test for influenza antibodies mentioned on p. 77; but in others, e.g. the method used by Findlay (1936) for his experiments on Rift Valley fever virus, the reverse procedure has been adopted, the amount of serum being kept constant and the virus diluted instead.

The relative value of these two methods cannot be assessed, but from the literature it would appear that, whilst certain of the virus-neutralizing antibodies may be made manifest by either of the methods described, in others the best results are to be obtained by adhering to one of them.

The criteria on which individual neutralization tests are interpreted vary greatly. In general they depend on the susceptibility of the animal used, e.g. in the case of neutralization tests with Rift Valley virus, as mice are employed for neutralization tests and as this virus is highly pathogenic to them, the results are interpreted according to the number of mice which survive. Should, however, a virus which is less infective to mice be employed, then after a definite interval of time all animals which do not die must be killed and examined for evidence of lesions. For example, in such tests with psittacosis, the spleen of each mouse must be stained and examined for the presence of elementary or inclusion bodies before it is possible to tell whether the serum has, or has not, neutralized the virus, and in the case of tests with influenza virus the lungs of the animals are searched for evidence of consolidation.

The mouse-protection test for yellow fever immune body is conducted on a different principle; thus before the serum-virus mixture is introduced intraperitoneally, the animal is given an intracerebral dose of sterile starch solution to induce the spread of virus from the brain capillaries to the brain itself, and the presence or absence of immune body in the serum tested is indicated according to the number of mice which develop fatal encephalitis.

With a certain number of viruses it has been possible to utilize cutaneous skin reactions for titrating the antibody content of immune serum, so that several experiments can be performed simultaneously on one animal.

Gordon (1925) used this method for his studies on vaccinia virus and we have used it for our experiments on rabbit myxoma and Shope's fibroma viruses (see van Rooyen, 1938; van Rooyen and Rhodes, 1938). The chorio-allantoic membrane of the developing chicken embryo can also be used for neutralization tests, and Burnet (1937, *et al.*, 1937) showed that after inoculating the serum-virus mixture on the membrane and counting the number of pocks produced, the neutralizing powers of the serum could be estimated (see p. 118). The technique of performing the mouse-protection test for the presence of yellow fever immune body is dealt with on p. 474. With regard to other tests of a similar nature, the following brief notes may be of value:

St. Louis

Wooley and Armstrong (1934) demonstrated the presence of virus-neutralizing antibodies in the sera of patients who had suffered from this disease. The brains of several infected mice were removed, and ground in a mortar with 0·85 per cent. saline of pH 7·6 to form a uniform suspension containing approximately 1 : 100 parts of brain tissue.[1] The mixture was centrifuged for 3 hours at 15,000 r.p.m., and the supernatant fluid removed and diluted 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000 in saline solution. Four conical flasks were next placed in a row and into the first of these were placed 0·3 c.c. of serum and 0·15 c.c. of 10^{-3} virus suspension; into the second were added 0·2 c.c. of serum and 0·1 c.c. of 10^{-4} virus suspension; into flask number three there were placed 0·2 c.c. of serum with 0·1 c.c. of 10^{-5} virus dilution; and in the fourth flask there were mixed 0·2 c.c. of serum and 0·1 c.c. of the 10^{-6} virus concentration. The mixtures were vigorously shaken, allowed to stand for 2 hours at room temperature, then 4 mice were inoculated intracerebrally with the contents of each flask, a dose of 0·03 c.c. being employed, so that 16 mice were injected for each serum tested.

With each series of experiments two control sera were included, the one (a positive) derived from a patient known to possess antibody and the other (a negative) serum obtained from a normal person. Before proceeding to inject the virus intracerebrally, as a precautionary measure, the sterility of the serum-virus mixtures was tested by inoculating agar plates and incubating them aerobically.

Interpretation of results.

The mice were observed for 14 days, and during this interval, if the number surviving was the same as in the case of the known positive control, then the serum examined was assumed to possess strong neutralizing properties. If, on the other hand, there were fewer survivors in the test serum series than in the positive control series, the serum was regarded as less strongly positive, and when the number of animals dying in the experimental and negative control series were equal the result was interpreted as inconclusive. Finally, when the number of deaths occurring in the tested serum exceeded those recorded in the negative control the result was regarded as negative. As an additional check on the test, the brains of mice which died were sectioned and examined for evidence of histological lesions indicating the presence of encephalitis.

Lymphocytic Choriomeningitis

Protective antibodies in the sera of men and monkeys after recovery from infection have been demonstrated by Wooley, Armstrong, and

[1] An average mouse brain was found to weigh approximately 0·30 gm.

Onstott (1937), who adopted the following method: Stock virus preserved in neutral glycerol and 0·85 per cent. sodium chloride solution at +5° C. was used for infecting 3 mice by intracerebral inoculation. One half of the brain of each animal was removed, ground in a mortar with 10 c.c. of 0·85 per cent. sodium chloride solution of pH 7·6, thoroughly mixed, then diluted to form 1 : 3,000, 1 : 5,000, and 1 : 10,000 concentrations respectively, and to 0·1 c.c. of each dilution was added 0·2 c.c. of the serum to be tested. The mixtures were well shaken, kept at 37° C. for 4 hours, and 0·03 c.c. from each serum-virus mixture was inoculated intracerebrally in a mouse, using 4 animals for each dilution, i.e. 12 mice for each serum examined.

Two serum controls were included in the experiment, the one known to possess neutralizing antibody against the virus, and the other a serum containing none.

Interpretation of results.

The mice were observed for a period of 14 days, and in the event of a greater or similar number of animals surviving among the test serum series, as compared with the known positive control, the serum examined was regarded as strongly positive. When the number of mice dying among the test serum group was the same as in the negative control group, the serum tested was considered as negative. Results intermediate to these were interpreted accordingly. In the case of mice which died, typical features of the experimental disease were ruffling of the hair, fine tremors and extension of the hind legs at time of death, accompanied by characteristic histological appearances in the brain (see Ch. LXVII).

Poliomyelitis

Kolmer and Rule (1935) recommended the following method for detecting the presence of virus-neutralizing antibody in human serum: The spinal cords from severely paralysed monkeys were removed, stored in 50 per cent. glycerol in the refrigerator for a month, and a saline suspension containing about 10 m.i.d. of virus per 0·5 c.c. was prepared as follows: one gm. of the spinal cord was washed and ground up in 20 c.c. of saline to make a 5 per cent. suspension, centrifuged at 1,500 r.p.m. for 3 minutes, and the supernatant fluid used for the experiment. Then 0·5 c.c. of this material was mixed with 0·5 c.c. of patient's serum, placed in the water-bath at 37° C. for 2 hours, and 0·5 c.c. injected intracerebrally in a *M. rhesus* monkey. The control test was carried out by inoculating another monkey with a similar suspension of virus, to which saline had been added instead of serum.

Interpretation of results.

If the control animal developed paresis in 6 to 9 days, and the test monkey remained unaffected, then it was assumed that the individual's serum possessed sufficient antibody to neutralize 10 m.i.d. of virus, and he or she was immune to the disease. In the case of a serum from which antibody was absent, both monkeys developed the disease with equal severity, and when results intermediate to these were recorded they were interpreted accordingly.

The Standardization of Antipoliomyelitis Convalescent Serum

The need for establishing a standard for assessing the potency of various specimens of human antipoliomyelitis convalescent serum has long been

recognized, and Madsen, Jensen, and Eagles (1936) have devised the following method:

The minimum completely paralysing dose (m.c.p.d.) of a 'standard' suspension of virus, consisting of the Flexner strain of virus stored in glycerol, was first estimated by titration. A 10 per cent. saline suspension was made from cervical or lumbar cord, centrifuged at 3,000 r.p.m. for 10 minutes, the supernatant fluid removed, and six dilutions of virus consisting of 0·1, 0·01, 0·001, 0·0001, 0·00001, and 0·000001 per cent. were prepared. Then 0·5 c.c. of each was introduced intracerebrally in a monkey, and the m.c.p.d. was estimated. For example, if paralysis only occurred in the 0·1, 0·01, and 0·001 per cent. virus suspensions, then the m.c.p.d. was taken as 0·5 c.c. of a 0·001 per cent. dilution of the 'standard' virus.

For the neutralizing tests 0·1 per cent. virus was usually employed, and this figure provided an adequate margin of infectivity.

Technique.

The serum to be examined was first tested undiluted and, if found potent enough to neutralize all the virus, it was subsequently diluted. 1 c.c. of serum was added to 1 c.c. of 1 per cent. virus (equivalent to 1,000 m.c.p.d.), thoroughly mixed, incubated at 37° C. for 2 hours, placed in a cold room overnight, and 1 c.c. of the mixture, consisting of 0·5 c.c. virus and 0·5 c.c. serum, injected intracerebrally in a monkey.

The control consisted of 1 c.c. saline plus 1 c.c. of 1 per cent. virus exposed to the same conditions.

Monkeys were kept under observation for 30 days, and in the case of the control, 1 per cent. virus always produced complete paresis within 12 days after inoculation.

The proposed standard for antipoliomyelitis convalescent serum (Madsen and Jensen, 1936).

During the 1934 epidemic of poliomyelitis in Denmark (Jensen, 1935), serum was stored by obtaining large quantities thereof from over a thousand convalescent subjects, passing it through a Seitz filter, and drying it in a Krause evaporator (see also Craigie, 1931). The dried product was then thoroughly mixed and regarded as a representative pooled sample of standard serum. The proposed standard was prepared as follows: 500 gm. of desiccated serum was dissolved in distilled water to a total of 5 litres, placed in the cold room for 24 hours, centrifuged at 10,000 r.p.m. for 1 hour, and passed through a Seitz filter. The filtrate was tested for sterility, distributed in 5 c.c. amounts into ampoules, desiccated *in vacuo* over phosphorous pentoxide, and then each ampoule was filled with pure dry nitrogen and sealed off.

Influenza

Tests for demonstrating the presence of neutralizing antibody for influenza virus with mice have been described by Laidlaw, Smith, Andrewes, and Dunkin (1935) and are conducted as follows: Several mice are infected with influenza virus, their lungs removed, ground in a sterile mortar to form a paste, triturated with saline to make up a 5 per cent. suspension of lung tissue, centrifuged, the supernatant fluid removed and clarified by passage through an asbestos and paper pulp filter, and then filtered through a 'gradocol' membrane of a.p.d. 0·9 μ. Equal quantities of the virus filtrate are added to 1:10, 1:50, and 1:250 dilutions of serum to be tested; the mixtures are then left for 10 hours at room temperature, and

thereafter 0·05 c.c. of each dilution is introduced intranasally in mice which have been lightly anaesthetized with ether. At the same time, a control test is also set up in which normal serum free from influenza antibody is substituted for immune serum. Five to seven days after inoculation all surviving mice are chloroformed, autopsied, and the presence or absence of lung lesions noted, from which the results of the test and control experiments are interpreted.

In subsequent experiments Andrewes, Laidlaw, and Smith (1935) added a refinement to their technique by titrating simultaneously with each human serum a standard horse antiserum, the neutralizing powers of which supplied a basis for comparison, so that the activity of human sera could be assessed in terms of the standard immune horse serum. The technique employed by Magill and Francis (1938) was similar to the above, with the exception that a standard strength of unfiltered centrifuged suspension of influenza virus was used, whereas Laidlaw, Smith, Andrewes, and Dunkin used filtered virus as they found that the intranasal inoculation of tissue particles was liable to cause lung abscesses in mice.

Rift Valley Fever

Neutralizing antibodies to this agent have been demonstrated in the sera of human beings recovered from an attack of the disease (Findlay, 1936).

Technique.

A 10^{-1} dilution of infected mouse liver was diluted with saline to form dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}; to each of these was added an equal volume of immune serum, and to a similar series of dilutions normal serum was added to serve as a control. The two sets of mixtures were incubated at 37° C. for 4 hours and 0·4 c.c. of each serum-virus mixture was inoculated intraperitoneally in mice. The results showed that an immune serum was able to neutralize a 1 : 10 or higher dilution of virus, whereas normal serum was incapable of doing so, and the animals accordingly succumbed to infection in 2 to 5 days' time.

During the course of these studies Findlay demonstrated the phenomenon of dissociation of apparently neutral serum-virus mixtures by simple dilution with saline solution. From his tests he proved that dissociation by dilution did not occur when the virus was treated with an excess of immune serum. It was not possible to influence the result by reducing the length of time for which the virus and immune serum were kept in contact; for example, the same results were recorded after 15 minutes as were obtained after 4 hours' preliminary incubation before inoculation. Reactivation of a neutral serum-virus mixture could, however, be produced by dilution provided that the serum and virus were mixed together in certain definite proportions, and it was shown that when the amount of virus was decreased, and the quantity of immune serum kept constant, a point was reached at which reactivation by dilution was no longer possible.

Another factor shown to affect the results was the route by which animals were inoculated—a point already illustrated in connexion with experiments on influenza virus by Francis and Magill (1935), who revealed that an apparently neutral serum-virus mixture, although devoid of infectivity when administered intraperitoneally, was pathogenic if introduced intranasally. Findlay made a similar observation in the case of the immunity mechanism of Rift Valley virus, and moreover, he conducted a series of experiments to determine whether this effect was exactly parallel to that of dissociation due to dilution. From his results he was able to

show that both these phenomena occurred in the same mixtures of immune serum and virus, and were therefore in all probability of the same character. It was also demonstrated that the dose inoculated had an important bearing on the problem, for a small dose of virus and immune serum mixture was found to be more pathogenic than a large inoculum thereof. This point served to explain why an apparently neutral serum-virus mixture when administered intraperitoneally was innocuous, but when introduced intranasally was lethal, since in a given time less of the mixture was absorbed through the nasal mucosa than via the peritoneum. In support of this opinion it was further shown that, whereas 0·4 c.c. of an apparently neutral serum-virus mixture injected intraperitoneally was inactive, a dose of 0·03 c.c. given by the same route proved to be fatal. Because of such results, Findlay concluded that the essential factor determining infectivity by different routes of inoculation (so far as these tests were concerned) was primarily the size of the dose, and not a greater infectivity because of the method of administration (see also Goyal, 1935).

Psittacosis

Bedson (1933) has demonstrated the presence of neutralizing antibodies in the sera of guinea-pigs immunized against psittacosis, and he later showed (1938) that such antibodies also appeared in the sera of inoculated mice. To perform the test, the sera of 8 animals were pooled and mixed with virus to produce a final concentration of 1,000 m.l.d. of the latter in 0·5 c.c. of the mixture. A control test was also set up, in which normal mouse serum was substituted for immune serum, and both series allowed to stand for 2 hours, when 4 mice were inoculated each with 0·1 c.c. of the control mixture and a similar number with each of three amounts, namely, 0·1, 0·5, and 1·0 c.c. of antiserum-virus mixture.

THE ABSORPTION OF VIRUS-NEUTRALIZING ANTIBODIES

Vaccinia and Herpes

Smith (1930) demonstrated that rabbit's testis infected either with vaccinia or herpes virus was capable of absorbing its homologous antibody from an immune serum, whereas normal rabbit kidney failed to absorb immune body from an antivaccinial serum.

The specificity of the phenomenon was demonstrated by cross-absorption experiments in which vaccinial testis failed to absorb herpetic antibody, and herpetic testis failed to absorb vaccinial antibody. It was also shown that each virus selectively absorbed its homologous antibody when added to a mixture of antivaccinial and antiherpetic serum. Vaccinial testis suspension, in which the virus was destroyed by heating to 58° C. or 60° C. for 1 hour, showed greatly reduced power of antibody-absorption.

Technique.

5 to 7 gm. of vaccinial testis was minced with scissors in a sterile mortar with 10 c.c. of a 1:10 dilution of immune serum and the suspension transferred to test-tubes; 5 to 7 gm. of normal testis (or kidney) was likewise treated and utilized as the control; 0·5 c.c. of pure anaesthetic ether was added to each tube which was hermetically sealed and stored for 3 days at 37° C., in order to destroy any contaminating bacteria present.

After 3 days the tubes were opened to allow the ether to evaporate, and were centrifuged at 3,000 r.p.m. for ½ to 1½ hours. The supernatant fluids were clarified by preliminary filtration through filter paper, or paper pulp,

and then passed through L_2 candles under a negative pressure of 40 cm. of mercury. The final filtrates thus represented (*a*) immune serum treated with testicular virus, and (*b*) immune serum treated with normal control tissue.

The method of antibody titration was as follows: in order to compare the antibody content of any two sera, various dilutions of virus (i.e. of a suspension of herpes or vaccinia virus contained in testis) were added to equal quantities of each of the sera; 0·2 c.c. of the mixtures was then injected intradermally in the shaved skin of a rabbit, using one half of the animal's back for each set of dilutions.

The relative distribution of inocula on the rabbit's skin may be represented schematically thus:

HEAD

A

Left side.		*Right side.*
Serum A (½ dil.)+virus (1/20,000 dil.)	0	0 Serum B (½ dil.)+virus (1/20,000 dil.)
,, ,, +virus (1/2,000 dil.)	0	0 ,, ,, +virus (1/2,000 dil.
,, ,, +virus (1/200 dil.)	0	0 ,, ,, +virus (1/200 dil.)
,, ,, +virus (1/20 dil.)	0	0 ,, ,, +virus (1/20 dil.)
Control of serum only	0	0 Control of serum only

B

TAIL

Interpretation of results.

Reactions were read on the third, fourth, and fifth days after inoculation. By measuring the size of the reaction produced by each dilution and the titre to which this occurred, it was possible to compare the virus-neutralizing properties of serum A with those of B.

Influenza

Absorption tests were utilized by Smith and Andrewes (1938) for their investigations into the complex antigenic structure of influenza virus, and they showed that each strain of virus only absorbed from a homologous serum those antibodies which corresponded to the antigenic components of the absorbing strain. Technical details regarding the method of performing the test were as follows: A dilution of serum capable of neutralizing 100 m.i.d. of virus was added to a 5 per cent. suspension of fresh infected mouse lung, and absorption allowed to take place by incubating the mixture at 37° C. for 2 hours followed by placing it in the refrigerator overnight. The excess unabsorbed virus was next eliminated, either by filtering the suspension through a collodion membrane possessing an a.p.d. of 0·1 μ which retained the virus, or else by heating the material to 55° C. for 30 minutes. The absorbed sera were now tested for the presence of virus-neutralizing antibodies by admixture with dilute virus filtrates of about 100 m.i.d. in potency, and the result was compared with serum which was unabsorbed but had been subjected to similar treatment. Each serum-virus mixture was incubated at 37° C. for 2 hours and thereafter tested for infectivity by intranasal inoculation of mice. Control tests were also incorporated in the series, and it was shown that the virus-neutralizing antibodies could not be absorbed by normal lung tissue. From these experiments Smith and Andrewes were able to show that absorption of a serum with a homologous strain of virus removed only its appropriate antibodies, leaving the others intact. The process is therefore analogous to the specific absorption of antibodies from an antibacterial serum by treatment with the homologous organism.

AGGLUTINATION OF ELEMENTARY BODIES

There has been a certain amount of discussion regarding the first worker to agglutinate the elementary bodies of vaccinia, and Gordon (1935, 1935*a*) states that Tanaka (1902) was the earliest to observe that a suspension of vaccine lymph underwent flocculation when brought into contact with immune serum. Later, Paschen of Hamburg demonstrated the phenomenon to a local biological society and prepared an illustration of the reaction for an article in Kraus and Levaditi's proposed book on technical methods, the publication of which was unfortunately cancelled on account of the Great War. Additional comments on this matter will be found in the notes of Ledingham (1935, 1937).

Vaccinia

Craigie (1932) has employed the following method for obtaining a suspension of vaccinial elementary bodies:

Technique of preparation of antigen.

A piece of bronze wire gauze (100 mesh) is used for scarifying the shaved back of a rabbit; a strip of the gauze (0·7 cm. by 10 cm.) is held with a pair of Kocher forceps, and the virus suspension is placed on the skin of the rabbit which is then inoculated by drawing the rough edge of the gauze over the animal's back. Three days later the rabbit is exsanguinated, killed, the skin removed, cleaned with ether, pinned out on a board, and the pulp scraped off with a scalpel into two successive 10 c.c. volumes of chilled citric acid (0·004 M) one part and phosphate buffer of pH 7·0 one part diluted 1:50 in distilled water.

The pulp suspension is not ground-up but is thoroughly shaken, centrifuged at 3,500 r.p.m. for 10 minutes, and the supernatant fluid removed and set aside. The deposit is once again washed in dilute buffer solution, and the supernatant fluids obtained after the first and second washings pooled. The mixture is now placed in flat angle centrifuge test-tubes of 4 mm. internal diameter, and centrifuged at 3,500 r.p.m. for 60 minutes at an angle of 28° from the vertical. The elementary bodies deposited at the end of this interval are resuspended in fresh buffer solution and subjected to two further washings. The final suspension is dispersed by thorough shaking and once more centrifuged at 3,500 r.p.m. for 10 minutes on the ordinary centrifuge, in order to remove coarse tissue particles. The supernatant fluid containing the elementary bodies is now removed, placed in a glass-stoppered bottle, excess anaesthetic ether added, and stored in the ice-chest until required. The ether can be removed before using by placing a small quantity of the suspension in a partial vacuum. Craigie and Wishart (1934*a*) state that the yield of Paschen bodies obtained from a rabbit in 15–20 c.c. of dilute buffer has, when fresh, an intradermal infective titre of $1 : 10^{-8}$.

The density of the elementary body suspension used as antigen is standardized to match the opacity of a kaolin suspension corresponding to tube No. 2 of the McFarlane standard. The tubes are compared with each other by placing them against a dark ground illuminated from behind at an angle of 45°.

Technique of macroscopic agglutination test.

A series of serum dilutions 0·25 c.c. in bulk are made in 0·85 per cent. sodium chloride containing 1:50 citric acid phosphate buffer of pH 7·0.

The elementary body suspension (*vide supra*) is diluted to twice the standard opacity in distilled water containing 1 : 50 of this buffer, and 0·25 c.c. added to each serum dilution. The mixtures are incubated in a water-bath at 50° C. for 16 hours and the results read macroscopically. By means of agglutinin-absorption tests Craigie and Wishart (1935) have demonstrated the existence of two types of agglutinins in antivaccinial sera. The two corresponding agglutinogens differ in their relative stability to heat and other agents. The more labile 'L.' agglutinogen has its agglutinability and ability to absorb agglutinin impaired or destroyed by exposure to a temperature as low as 56° C. The 'S.' agglutinogen, on the other hand, is thermostable even after heating to 95° C. (see Craigie and Wishart, 1934*a*, 1935; also p. 335).

Zoster and Varicella

Amies (1934) has demonstrated the relationship of these two viruses to one another by means of cross-agglutination reactions.

Antigen.

Vesicle fluid was collected from patients, not more than 60 hours after the beginning of vesiculation, with a sterile capillary pipette. It was diluted in physiological saline containing 2 per cent. sodium citrate, centrifuged at low speed to deposit tissue cells, then the supernatant fluid was removed and recentrifuged at 12,000 r.p.m. for 30 minutes.

The deposit (which was frequently invisible to the naked eye) was re-suspended in saline of pH 7·0 containing 0·25 per cent. formalin. After a final fractional centrifugation at low speed to throw down gross particles, the supernatant fluid was removed and the suspension standardized to approximate in density that of an arbitrarily chosen elementary body suspension.

Technique of microscopic agglutination test.

The hanging drop method was used. Dilutions of convalescent serum derived from cases of zoster and varicella were prepared from 1 : 4 to 1 : 128 in saline, mixed with an equal volume of antigen, and placed on a coverslip which was inverted over a glass slide fitted with a metal ring. The mixtures were left at room temperature (average 65° F.) for 24 to 48 hours and then examined under the microscope, using a 2/3 in. objective and ×10 ocular for evidence of agglutination. A positive test was signified by the presence of highly refractile, irregularly shaped clumps.

Agglutination was found to occur between zoster elementary bodies and homologous sera up to an end-titre of 1 : 128, and in the case of varicella up to a similar endpoint. Cross-agglutination was less marked, thus a 1 : 64 dilution of zoster sera agglutinated varicella elementary bodies, whereas zoster elementary bodies reacted with 1 : 128 dilution of zoster serum. Varicella sera, on the other hand, agglutinated varicella elementary bodies up to a 1 : 128 dilution and also zoster bodies to a similar titre. It may here be noted that results opposite to these have recently been reported by Hasskó *et al.* (1938), who employed complement-fixation reactions and found that the viruses of zoster and varicella are not identical—although they may be antigenically related.

Psittacosis

Bedson (1932) has demonstrated that the elementary bodies found in this disease can be agglutinated by specific antisera. Antigen was prepared from infected mouse spleen by fractional centrifugation, and the

elementary bodies were deposited after the final washing by centrifuging the suspension for 2 hours at 5,000 r.p.m.

The antiserum employed consisted of the sera of guinea-pigs which had been immunized with psittacosis virus obtained from infected guinea-pig tissues. Two control sera were also used, the one consisting of the serum of a guinea-pig which had been inoculated with mouse spleen tissue, and the other antiherpetic serum also of guinea-pig origin. The hanging drop technique was used for the tests and the results were read after incubating the mixtures at 37° C. for 24 hours. From these experiments, Bedson proved that a suspension of psittacosis elementary bodies could only be agglutinated by its homologous antiserum, and not by herpetic antiserum nor by antiserum to normal mouse spleen.

PRECIPITIN REACTIONS

A number of these reactions have been described and the soluble substances of certain viruses, e.g. vaccinia, yellow fever, Shope's rabbit fibroma, and myxoma (Rivers and Ward, 1937), have been shown to react specifically with their homologous antisera.

Vaccinia

The following products of vaccinia virus have been proved to be precipitable with antivaccinial serum: A precipitable substance in Seitz (EK) filtrates of vaccinial pulp divisible into two antigenic fractions consisting of a thermolabile (L.) portion which is destroyed by heating at 56° C. for 1 hour, and a thermostable (S.) component which resists 95° C. for a similar period.

Both these antigens occur in the elementary bodies of vaccinia and participate in the agglutination of these bodies with vaccinial antiserum (Craigie, 1932; and Craigie and Wishart, 1936 *a*). The L.S. fraction is prepared as follows:

Technique.

A Seitz (EK) filtrate is dialysed in cellophane bags. A 1/10 volume of Sörensen's hydrochloric acid-citrate buffer (Clark, 1928) of pH 4·45 is added to the dialysed filtrate and the mixture centrifuged for 30 minutes. The deposit is drained and the fluid adhering to the walls of the tube dried with filter paper. The deposit is then suspended in Sörensen's citrate caustic soda buffer of pH 6·5, the volume used being 1/10 of the final volume desired. Distilled water is added to the amount of four times this volume, the tubes shaken, centrifuged, and the supernatant fluid set aside. The deposit is extracted a second time with the same volume of buffer (pH 6·65) and distilled water as previously used. The two supernatants are pooled, extracted with ether, kept for 24 to 48 hours in the ice-chest, recentrifuged to deposit large particles, the supernatant fluid removed and used as L.S. fraction.

The antigenic properties of the L. and the S. components have been demonstrated by immunizing rabbits with each of them. The sera thus obtained react specifically to produce a precipitate with the homologous fraction in each instance. It has been pointed out that, whereas 56° C. annuls the *in vitro* activity of the L. component, in order to abolish its *in vivo* antigenic effect towards rabbits it must be heated to 70° C. for 1 hour. The antigenic properties of the S. component, on the other hand, are unaffected after heating at 90° C. (Wishart and Craigie, 1936).

A third precipitable substance has been obtained by extraction of

elementary bodies after they have been freed from other tissue products by repeated washing. This has been termed 'dissociated antigen' since it is liberated from elementary bodies *in vitro*. It also consists of thermolabile and thermostable soluble precipitable substances, both of which are similar to the soluble precipitable substances found in suspensions of fresh vaccine pulp (Craigie and Wishart, 1936 *b*).

Parker and Rivers (1935, 1936, 1937) have confirmed Craigie and Wishart's findings. They succeeded in extracting a chemically stable, serologically active substance from dermal and testicular tissue infected with vaccinia by boiling it with 50 per cent. ammonium sulphate, followed by dialysis against running water, precipitation with alcohol, and treatment with buffered solutions. About 15 mg. of a white substance was derived from about 200 c.c. of an extract of dermal vaccine virus, and this was susceptible to digestion with commercial trypsin or pepsin but resistant to crystalline trypsin and chymotrypsin, and possessed the characters of an alcohol-soluble protein which was not precipitated by boiling in a neutral aqueous solution. A dilution of 1:640,000 gave a precipitate with an antivaccinial S. antigen serum (Craigie and Wishart, 1934 *a*), but produced no reaction in a serum from which the S. agglutinin had previously been removed by absorption. The chemical composition of washed elementary bodies has been investigated by Hughes, Parker, and Rivers (1935), who found that they contained ash, carbohydrate, fat, and nitrogen, part of which was in the form of protein. In general, the chemical structure of elementary bodies was similar to that of bacteria and other material of a protein nature.

Yellow Fever

Hughes (1933) showed that the sera of monkeys which had recovered from the disease developed a precipitin capable of reacting with a precipitinogen occurring in the blood of monkeys during the acute phase of the disease. This precipitinogen, which was associated with the albumen fraction of the serum, was not the virus of yellow fever *per se*. Its amount varied with the severity of the infection and it was independent of the virus-neutralizing antibody; it disappeared after the monkey recovered from the disease, and was thought to be responsible for stimulating the development of precipitating antibodies in the sera of animals after an attack of yellow fever.

A similar precipitin was found in convalescent human sera, which reacted with the precipitinogen occurring in the blood of infected monkeys.

Technique.

Serum derived from a monkey moribund from yellow fever was filtered through a Seitz filter, and antiserum obtained from a monkey which had recovered from a virulent (Asibi) strain of yellow fever was likewise treated. The two were then mixed in a test-tube, incubated at 37° C. for 2 hours, and examined for the presence of cloudiness. The ring technique was also tried by putting undiluted antiserum in the foot of a test-tube (2 mm. in diameter) with the aid of a capillary pipette, and this was overfloated with serum derived from an ill animal. The two fluids were next incubated at 37° C. for 2 hours and the results thereafter read in artificial light with the aid of a dark background. A positive result was signified by the formation of a ring of turbidity at the fluid interface, while in the control test, in which normal monkey serum was substituted for antiserum, no such reaction occurred.

Nicht einzeln im Buchhandel. *Ueberreicht vom Verfasser.*

Sonderabdruck aus dem

Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten.

I. Abteilung. Originale.

1938. Bd. 142.

Verlag von Gustav Fischer in Jena.

Printed in Germany.

[From the Dept. of Bacteriology, Edinburgh University, Scotland.]

The immunological relationship of Shope's rabbit fibroma virus to the virus of infectious myxomatosis: complement fixation studies.

By **C. E. van Rooyen and A. J. Rhodes.**

Infectious myxomatosis of the rabbit is usually a fatal disease accompanied by highly characteristic morbid anatomical and histological features. Shope's fibroma, on the other hand, is a benign affection, particularly in the British domestic rabbit, in which it is seldom fatal. Notwithstanding the gross differences between these two conditions, recent work has revealed that some degree of immunological and other relationship exists between their aetiological agents.

For example, Berry and Dedrick (1936) successfully transformed the fibroma virus into myxoma by inoculating rabbits with a mixture consisting of live fibroma virus and heat-killed myxoma virus. This important discovery has recently received the independent confirmation of Hurst (1937).

The sera of rabbits recovering from infection with Shope's fibroma virus have been demonstrated to contain precipitins for myxoma virus, and vice versa (Rivers and Ward, 1937). Likewise the presence of cross-agglutinins has been described by Ledingham (1937). All the data therefore, point to the undoubted biological relationship of these two viruses, notwithstanding the entirely different pathological lesions they produce.

The object of the present investigation has been twofold; first to find out whether the sera of rabbits immunized against myxoma virus contained complement-fixing antibodies for that virus, and if so, whether the reaction was specific for myxoma or whether it occurred also with fibroma virus. Second, the converse was also investigated, Shope fibroma antiserum containing complement-fixing antibodies for that virus (see van Rooyen, 1938) was examined for cross complement-fixation with myxoma antigen.

Immunization of rabbits.

Three different methods were used to immunize rabbits against myxoma virus, two of these (a and b) as employed by earlier workers, and (c) a method devised by ourselves.

(a) Rabbits were inoculated with Shope's fibroma virus, allowed to recover, and two to three months later they were injected with a 1 : 10000 suspension of myxoma virus (desiccated infected rabbit's testicle). Not infrequently, some of the animals developed myxoma and died, but those which recovered were given subcutaneously 0.5 cc. of a further dose of a 1 : 1000 dilution of myxoma virus.

(b) Rabbits which had recovered from the fibroma infection were inoculated with 0.5 cc. of 1:10000 dilution of myxoma "neurovirus", which had been passed 28 times serially through the brains of rabbits (see Rhodes, 1938). Occasionally a few of these animals developed myxoma, but more usually, "neurovirus" proved to be less fatal than testicular passage material. Animals which recovered received a further subcutaneous dose of 0.5 cc. of a 1:1000 dilution of the myxoma virus (testicular material).

(c) Rabbits which had been successfully immunized against myxoma by procedures (a) or (b) were bled, and 1.5 cc. of serum added to 0.25 cc. of a 1:1000 dilution of myxoma "neurovirus". This mixture was incubated at 37° C. for four hours and 0.5 cc. was introduced subcutaneously into a normal healthy rabbit. In many instances the myxoma antiserum proved to have effectively neutralized the myxoma "neurovirus", for the mixture proved to be non-infective. Later, such animals withstood a subcutaneous dose of 0.5 cc. of a 1:1000 dilution of virulent virus (testicular material).

The relative merits of these three methods may be summarised by stating that wheras there was no difference in the degree of resistance to reinfection produced by them, procedure (c) was obviously the most satisfactory for our cross-complement fixation studies. Its obvious advantage lay in the fact that rabbits immunized by this method were uninfected with Shope fibroma virus at any stage. In the case of animals immunized by procedures (a) and (b) the rabbits were bled before inoculation with Shope's fibroma virus as well as two, three, four and twelve weeks later. At this stage, the animals were injected with myxoma virus. Two weeks later, when the second dose of myxoma virus had been given, another specimen of serum was removed. A final bleeding was made eight weeks later. The serum was inactivated at 55° C. for 30 minutes. About 1 cc. of each specimen was used for the immediate complement-fixation test (done on the same day). The remainder was sealed and stored in the refrigerator, to be available for tests at a later date. Rabbits immunized by method (c) were bled before inoculation, and also two, three, four, twelve, fourteen and twenty weeks later.

Technique of complement-fixation tests.

A fixed volume of serum and varying amounts of complement have been employed in these tests. The degree of positivity of the reaction has thus been assessed in terms of the number of units of complement fixed.

Reagents.

Serum. A quantity of 0.05 cc. of inactivated rabbits serum obtained as described above was used throughout.

Antigen. Desiccated infected rabbits testis was diluted 1:1000 in 0.85% sodium chloride solution, centrifuged at 3000 r. p. m. for 30 mins., and the supernatant fluid discarded. The deposit was suspended in saline and the opacity of the suspension standardised approximately to a Brown's opacity tube No. 4. In practice, this was found to give the maximum degree of opalescence without interfering with visual interpretation of lysis.

Complement. Guinea pig serum preserved by Sonnenschein's (1930) method (see Mackie and McCartney, 1938), was used throughout. This was titrated on each occasion, before use, the minimum haemolytic dose (m. h. d.) varying generally from 0.005 to 0.006 cc.

Haemolytic system. Sheep erythrocytes were washed thrice in 0.85 per cent saline and a 3 per cent suspension made in saline. The cells were

sensitised with a standard glycerinated sheep immune body (B. W. & Co.), usually 0.35 cc. sensitising 50 cc. of a 3 per cent suspension of sheep cells.

Procedure. For the test, four tubes containing 0.05 cc. of serum were placed in a row, 0.5 cc. of antigen added to each tube, and followed by 4, 6, 8 and 10 m. h. d. of complement respectively.

Controls. The serum controls consisted of two tubes containing 0.05 cc. of serum, 4 and 6 m. h. d. of complement and 0.5 cc. of saline. The antigen controls comprised two tubes with 0.5 cc. of antigen and 4 and 6 m. h. d. of complement respectively. As previously described, the antigen consisted of a saline suspension of rabbit testis infected with either Shope fibroma or myxoma virus.

Length of time for which mixtures were incubated. The above mixtures were incubated at 37° C. for 1½ hours, and then 0.5 cc. of haemolytic system was added. The tubes were then re-incubated at 37° C. for 1½ hours, removed from the incubator and the result read immediately. A final reading was made after the test had stood for 24 hours at room temperature.

Interpretation of results.

The presence of unlysed (sedimented) red-cells signified fixation of the particular amount of complement in that tube. Any degree of fixation less than 4 m. h. d. was ignored, as it was occasionally noted that normal rabbit serum yielded a fixation of 2 or 3 m. h. d. with virus antigen.

Table I indicates the results obtained with a series of rabbits after they had been immunized first against Shope virus, and then with myxoma virus.

Table I.

Rabbits immunised by procedures (a) and (b), against:

M. H. D.	Before immunisation	Shope fibroma virus						myxoma virus			
		Weeks after inoculation						Weeks after inoculation			
		2.		3.		4.		12.		14		20	
	Sh.	Myx.	Sh.	Myx.	Sh.	Myx.	Sh.	Myx.	Sh.	Myx.		Sh.	Myx.	Sh.	Myx.
4	—	—	+	—	++	—	++	—	—	—	Inoculated with myxoma virus (two doses)	—	++	—	+++
6	—	—	—	—	+	—	++	—	—	—		—	++	—	++
8	—	—	—	—	—	—	+	—	—	—		—	—	—	++
10	—	—	—	—	—	—	—	—	—	—		—	—	—	+
Serum-control 4	—	—	—	—	—	—	—	—	—	—		—	—	—	—
Serum-control 6	—	—	—	—	—	—	—	—	—	—		—	—	—	—
Antigen-control 4	—	—	—	—	—	—	—	—	—	—		—	—	—	—
Antigen-control 6	—	—	—	—	—	—	—	—	—	—		—	—	—	—

+++ = complete absence of lysis.
++ = partial lysis.
+ = slight lysis.
— = complete lysis.
Sh. = Shope fibroma virus antigen.
Myx. = myxoma virus antigen.

The serum of each animal was tested for complement-fixing antibodies before they had been immunized, and 2, 3 and 4 weeks after the date of the first injection. The result of the test 8 weeks after the rabbit received the last dose of fibroma virus or twelve weeks after the first dose, are recorded in the column headed 12.

Rabbits now received two injections of myxoma virus, and this was followed by further tests carried out at the 14th and 20th week.

Reprinted from
The British Journal of Experimental Pathology,
1938, Vol. XIX, p. 156.

SPECIFIC COMPLEMENT-FIXATION WITH SHOPE'S FIBROMA VIRUS AND ITS RELATIONSHIP TO VIRUS-NEUTRALIZING PROPERTIES OF IMMUNE SERA.

C. E. VAN ROOYEN.

From the Department of Bacteriology, University of Edinburgh.

LONDON: H. K. LEWIS & CO. LTD., 136 GOWER STREET, W.C. 1.

Reprinted from the British Journal of Experimental Pathology, 1938, Vol. XIX, p. 156.

SPECIFIC COMPLEMENT-FIXATION WITH SHOPE'S FIBROMA VIRUS AND ITS RELATIONSHIP TO VIRUS-NEUTRALIZING PROPERTIES OF IMMUNE SERA.

C. E. VAN ROOYEN.

From the Department of Bacteriology, University of Edinburgh.

Received for publication February 24th, 1938.

THE existence of specific complement-fixing antibodies has already been demonstrated in the sera of human subjects and in animals following recovery from certain virus infections.

The present paper records the development of specific complement-fixing antibodies in the sera of rabbits after immunization with Shope's fibroma virus.

METHODS.

Strain of virus.—Two varieties of this virus have been used in the experiments, namely, the inflammatory (I A) strain and the fibromatosis (O A) type.

Method of immunization.—1 gm. of desiccated rabbits' testis infected with the I A strain of virus active to an end-titre of 1 : 1,000,000 dilution in broth was used as antigen. This was ground in a mortar, emulsified in 9 c.c. of 0·86 per cent. saline, centrifuged at 3000 r.p.m. for 30 min., the supernatant fluid removed, and used for immunization as live virus. A similar 1 : 10 dilution of infected tissue was prepared, heated to 55° C. for 60 min., tested for non-pathogenicity and used for immunization. Rabbits inoculated subcutaneously with this extract failed to show any cutaneous reactions, and the virus was therefore presumed to be inactive or dead.

The antigen control consisted of a 1 : 10 dilution of normal desiccated rabbits' testicle which was used for injecting control animals.

Twelve rabbits were immunized with live virus, a similar number with inactive (heated) virus and 6 with normal rabbits' testicle tissue. Animals were injected at weekly intervals, and each received 0·5 c.c. subcutaneously, 1 c.c. intravenously, 2 and 4 c.c. intraperitoneally respectively.

Sampling of blood.—Sample bleedings were made at 2, 3 and 4 weeks after the date of the first injection, and a final specimen was taken 8 weeks after the last inoculation of virus. The serum was heated at 55° C. for 30 min. and about 1 c.c. used for an immediate complement-fixation test, the remainder being stored in the refrigerator.

Technique of complement-fixation tests.—A fixed volume of serum and antigen and varying amounts of complement have been employed in the tests. The degree of the reaction has thus been assessed in terms of the number of m.h.d. of complement fixed.

Reagents.

Serum.—0·05 c.c. of rabbits' serum previously heated to 55° C. for 30 min. obtained as described above was used.

Antigen.—A 1 : 1000 dilution of desiccated rabbits' testicular tissue was made up in 0·86 per cent. sodium chloride solution, centrifuged at 3000 r.p.m. for 30 min. and the supernatant fluid removed. The opalescence shown by different specimens of testis tended to vary, and it was arbitrarily decided to standardize the suspension to the density of Brown's opacity tube No. 4. In practice this was found to be the maximum degree of opalescence permissible without interfering with the reading of hæmolytic effects on the mixture.

Complement.—Guinea-pigs' complement which had been preserved after the method described by Sonnenschein (1930) was used throughout. This was titrated on each occasion before use, and the minimum hæmolytic dose (m.h.d.) usually varied from 0·005 to 0·006 c.c.

Hœmolytic system.—A 3 p.c. saline suspension of thrice washed sheep erythrocytes was sensitized with a standard amboceptor (B. W. & Co.), 0·35 c.c. being used to sensitize 50 c.c. of the suspension.

Procedure.—To 4 tubes containing 0·05 c.c. of serum were added 4, 6, 8 and 10 m.h.d. of complement respectively, followed by 0·5 c.c. of antigen to each tube. The effect of adding antigen last was also tried in a number of tests, and found to make no difference.

Control.—The serum control consisted of 2 tubes containing 4 and 6 m.h.d. of complement plus 0·5 c.c. of saline. The antigen controls comprised 2 tubes with 0·5 c.c. of antigen and 4 and 6 m.h.d. of complement respectively.

Length of time for which mixtures were incubated.—The above mixtures were incubated at 37° C. for 1½ hours, and then 0·5 c.c. of sensitized cells was added to each. The tubes were next re-incubated at 37° C. for 1½ hours, removed from the incubator and the result read. A final reading was made the next day after the tubes had been standing for 24 hours at room temperature.

Interpretation of results.—Although certain sera with antigen fixed as much as 8 m.h.d. of complement, the reaction as a whole was a weak one. This was probably due to the fact that only dilute semi-opalescent extracts of Shope testis could be used as antigen, more concentrated tissue suspensions masking hæmolysis. Fixation of less than 4 m.h.d. complement was ignored since normal rabbit serum sometimes fixed 2 or 3 m.h.d.

RESULTS.

Table I gives the results obtained with sera of rabbits which had been repeatedly immunized with living I A Shope virus.

The serum of each animal was tested for complement-fixing antibodies before they had been immunized, and 2, 3 and 4 weeks after the date of the

first injection (see columns 2, 3, 4). The results of the tests 8 weeks after the rabbits received the last dose of virus (or 12 weeks after the first dose) are also recorded.

Tests with two control sera were incorporated in each series ; one serum was obtained from a healthy uninoculated rabbit, and the other from a rabbit which had received four repeated injections of normal rabbits' testicular extract.

TABLE I.

Rabbit B.

	MHD complement.	Before immunization.	Weeks after first injection. 2.	3.	4.	After interval of 8 weeks. 12.
	4	—	+	++	++	—
	6	—	—	—	—	—
	8	—	—	—	—	—
	10	—	—	—	—	—
Serum control	4	—	—	—	—	—
	6	—	—	—	—	—
Antigen control	4	—	—	—	—	—
	6	—	—	—	—	—

Rabbit E.

	MHD complement.	Before immunization.	Weeks after first injection. 2.	3.	4.	After interval of 8 weeks. 12.
	4	—	+	+++	+++	..
	6	—	—	++	++	—
	8	—	—	+	+	—
	10	—	—	—	+	—
Serum control	4	—	—	—	—	—
	6	—	—	—	—	—
Antigen control	4	—	—	—	—	—
	6	—	—	—	—	—

— = complete lysis. + = partial lysis. ++ = trace of lysis. +++ = no lysis.

From the above and other data it was concluded that rabbits repeatedly injected with live Shope (I A) strain of virus develop complement-fixing antibodies for that agent. These were occasionally observed as early as two weeks after the first injection of live virus had been administered (Rabbit B), but were more usually present 3 weeks after the first inoculation, and were well marked at the fourth week (Rabbit E). In Rabbit A and 3 others which are not included in the table, notwithstanding repeated doses of live virus, no complement-fixing antibodies appeared, and in animal B only 4 m.h.d. were fixed. All 4 animals showed a cutaneous reaction after the first dose of virus had been given, and failed to show any further skin response when injected with active virus after their course of immunization. Thus, although the

animals appeared to have developed active immunity to Shope virus, it is interesting to record that no complement-fixing antibodies were demonstrable in their sera. The sera of these animals were later examined for virus-neutralizing properties and reference to these is made below.

Result of Immunization with Heated (Killed) Virus.

As stated earlier, 12 rabbits were inoculated with testicular extract containing Shope virus I A strain, which had been previously heated to 55° C. for 60 min. As before, sample bleedings were made from the animals before they were inoculated, and after 2, 3, 4 and 12 weeks respectively. Sera were tested for the presence of complement-fixing antibodies as in the case of rabbits which had been immunized with the live virus. The results are tabulated below.

TABLE II.

	Rabbit H.						
MHD complement tested.	Control serum before immunization.	Weeks after first injection. 2.	3.	4.	After interval of 8 weeks. 12.	Serum control.	Antigen control.
4	—	—	+	++	—	—	—
6	—	—	+	++	—	—	—
8	—	—	—	+	—	..	..
10	—	—	—	—	—	..	..

The foregoing result, noted in rabbit H, was very similar to the response elicited in three others. These indicated that the inoculation of heated (presumably killed) virus stimulated the production of specific complement-fixing antibodies. Their occurrence, however, was slightly delayed in comparison to the response following injection of live virus, and they did not appear until the third week. Their presence was well marked at the fourth week, and two months after the last dose of heated virus they had completely disappeared. In two rabbits not mentioned in the table, no complement-fixing antibodies could be demonstrated at any period.

Cross Complement-fixation Tests with I A and O A Strains of Virus.

Tests were performed in order to discover whether the sera of rabbits which had been immunized with I A strain of virus would fix complement in the presence of antigen consisting of a saline extract of testicle infected with the O A virus.

The serum of each animal was accordingly tested against I A and O A testis antigens.

Two serum controls were also included, one derived from a normal rabbit and the other from one which had been repeatedly injected with normal rabbit testis.

TABLE III.

Serum of rabbit injected with living Shope fibroma virus strain I A with—

M.H.D. of complement.	I A. B	I A. A	O A. B	O A. A	Normal testis. B	Normal testis. A	Saline.
4	—	++	—	++	—	—	—
6	—	+	—	+	—	—	—
8	—	+	—	—	—	—	..
10	—	—	—	—	—	—	..

Serum of rabbit injected with heated Shope fibroma virus strain I A with—

M.H.D. of complement.	I A. B	I A. A	O A. B	O A. A	Normal testis. B	Normal testis. A	Saline.
4	—	++	—	++	—	—	—
6	—	+	—	+	—	—	—
8	—	+	—	—	—	—	..
10	—	—	—	—	—	—	..

M.H.D. of complement.	Serum of normal rabbit with— I A.	O A.	Normal testis.	Saline.	Antigen control. I A.	O. A.	Normal testis.
4	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—
8	—	—	—	—	..	..	..
10	—	—	—	—	..	..	..

B = Before immunization. A = after immunization 2 weeks after the last dose.

The results indicated that the sera of rabbits which have been immunized with the I A strain of virus (live or heated) show cross complement-fixation for both the I A as well as the O A virus, though to a lesser extent. It was also possible to show that rabbits inoculated with the O A strain revealed similar cross complement-fixation for I A antigen. There was no evidence that the sera of rabbits immunized against Shope virus produced non-specific complement-fixation with either normal testis or that containing vaccinia virus.

The Relationship of Complement-fixing Antibodies to Virus-neutralizing Properties of Immune Sera.

A number of rabbits which had been immunized with the I A strain of virus were bled and their sera tested for virus-neutralizing antibodies. The group included animals which had been immunized with (*a*) live virus, (*b*)

TABLE IV.—*Diameter of Erythematous Reaction Produced by Injection of Diluted Virus Material.*

Sera tested.	Skin reaction after—	1 : 20.	1 : 200.	1 : 2000.	1 : 20,000.	1 : 2,000,000.
		(cm.)	(cm.)	(cm.)	(cm.)	(cm.)
Serum of rabbit (A) before immunization with live Shope virus (I A strain) Complement fixed with Shope virus <4 MHD	5 days	2	1·5	1	—	—
	10 ,,	2	1	0·5	—	—
Serum of same rabbit (A) AFTER immunization with live Shope virus I A strain Complement fixed with Shope virus <4 MHD	5 days	—	—	—	—	—
	10 ,,	—	—	—	—	—
Serum of rabbit (B) before immunization with live Shope virus I A strain Complement fixed with Shope virus <4 MHD	5 ,,	2	1·5	1	0·5	—
	10 ,,	2·5	1·5	1	0·7	—
Serum of rabbit (B) AFTER immunization with live Shope virus I A strain Complement fixed with Shope virus —4 MHD	5 ,,	1	—	—	—	—
	10 ,,	1·5	0·5	—	—	—
Serum of rabbit (E) before immunization with heated Shope virus I A strain Complement fixed with Shope virus <4 MHD	5 ,,	2	1·5	1	0·5	—
	10 ,,	2·5	2	1·5	1	—
Serum of rabbit (E) AFTER immunization with heated Shope virus (I A strain) Complement fixed with Shope virus = 10 MHD	5 ,,	0·5	—	—	—	—
	10 ,,	0·5	—	—	—	—
Serum of rabbit (K) before immunization with live Shope virus (I A strain) Complement fixed with Shope virus <4 MHD	5 ,,	1·5	1	1·5	—	—
	10 ,,	2	0·5	1	0·5	—
Serum of rabbit (K) AFTER immunization with live Shope virus I A strain Complement fixed with Shope virus = 8MHD	5 ,,	0·5	—	—	—	—
	10 ,,	0·75	—	—	—	—

heated (killed) virus, (*c*) those which showed complement-fixing antibodies in their sera, and (*d*) those in which no complement-fixing antibodies were demonstrable, notwithstanding the repeated injections of virus they had received (*e. g.* Rabbit A). The control consisted of serum obtained before immunization.

This experiment made it possible to estimate the degree of virus neutralization by a serum and to correlate this result with the complement-fixing power.

Technique.—0·25 c.c. of serum, heated to 55° C. for 30 min., was added to 0·25 c.c. of falling decimal dilutions of virus ranging from 1 : 20 to 1 : 2,000,000. The mixtures were thoroughly shaken, and each dilution was inoculated into the shaved skin of a rabbit. Two sets of titrations were performed on each animal, and the size of the papule was measured on the fifth and tenth days after inoculation.

The results indicate that after repeated injections of either living or killed virus the serum of the animal acquires neutralizing properties for the virus, and that the activity is unrelated to the complement-fixing power. For example, Rabbit A before immunization possessed neither complement-fixing antibodies nor virus-neutralizing properties in its serum, but after immunization with live virus, although no complement-fixing antibodies were developed, the serum of this rabbit had strong neutralizing power.

Shope fibroma virus immune sera thus could be showed to possess virus-neutralizing properties without evidence of specific complement-fixing antibodies. It has not been possible, however, to obtain sera containing complement-fixing properties without also possessing virus neutralizing properties. To summarize, it may be stated that complement-fixing antibodies may or may not appear in the serum of an animal immunized with Shope fibroma virus, but when present, the serum also possesses virus-neutralizing properties. The latter property has been demonstrated following inoculation with either living or dead virus, and may occur in marked degree without the serum exhibiting any evidence of specific complement-fixation.

DISCUSSION.

The facts recorded are plain, and are in agreement with similar findings of earlier workers in respect of other virus diseases. The present work has shown that complement-fixing antibodies appear in the sera of rabbits immunized either with living or dead Shope fibroma virus. At one time it was thought that dead viruses were devoid of antigenic value. This opinion has been dispelled by the work of Bedson (1931), who clearly demonstrated the immunizing properties of killed herpes virus. He moreover showed that whereas a suspension of virus material killed by heating at 60°–100° C. lost its antigenic power, it did not do so if formalin was added to the suspension before heating it. The immunizing value of killed virus has also been demonstrated in the case of influenza by Andrewes (1937), Wilson Smith (1937) and Fairbrother and Hoyle (1937). Both complement-fixing and virus-neutralizing properties developed in the sera of rabbits after repeated doses of either live or heat-killed Shope fibroma virus. It is interesting to record that although some of the

rabbits showed virus-neutralizing antibodies in their sera, no complement-fixation could be demonstrated at the same time. The converse, however, was not encountered, for every serum possessing complement-fixing antibodies also in high dilution neutralized the virus. The former were usually demonstrable 2–3 weeks after the first injection, and generally disappeared about 8 weeks after the last dose of virus had been administered.

Bedson (1933), on the contrary, working with psittacosis virus found that although convalescent patients' sera contained complement-fixing properties, it possessed a low virus-neutralizing effect. These facts tend to suggest that the immunity mechanism prevailing in psittacosis infection might be different from that produced by Shope fibroma virus.

CONCLUSIONS.

1. Rabbits immunized with live Shope fibroma virus of either the I A or O A strain developed complement-fixing as well as virus-neutralizing properties in their sera.

2. Both the I A and O A strains were indistinguishable from their complement-fixation reactions.

3. Complement-fixing antibodies appeared about 2–3 weeks after injection. They usually disappeared about 8 weeks after the last dose of virus was given.

4. Shope fibroma virus when heated to 55° C. for one hour still retains its antigenic properties, giving rise to both complement-fixing and neutralizing antibodies.

5. Notwithstanding repeated doses of living (or dead) virus, no complement-fixing antibodies appeared in the sera of certain rabbits.

6. The sera of such animals, however, showed a powerful virus-neutralizing effect.

7. Whereas all the sera yielding complement-fixation also neutralized the virus, none were found in which complement-fixing antibodies were present without showing virus neutralization also.

It is with pleasure that I desire to thank Dr. C. H. Andrewes, who kindy supplied me with specimens of Shope fibroma and papilloma virus. Also Prof. Mackie for many helpful suggestions received. The work has been done during receipt of an expenses grant from the Medical Research Council.

REFERENCES.

ANDREWES, C. H.—(1937) *Brit. Med. Journ.*, **2**, 513.
BEDSON, S. P.—(1931) *Brit. J. exp. Path.*, **12**, 254.—(1933) *Ibid.*, **14**, 162.
FAIRBROTHER, R. W., AND HOYLE, L.—(1937) *J. path. Bact.*, **44**, 213.
SMITH, W.—(1937) *St. Mary's Hosp. Med. Gaz.*, **43**, 112.
SONNENSCHEIN, C.—(1930) *Zeit. f. ImmunForsch.*, **67**, 512.

Made and printed in Great Britain for
H. K. Lewis & Co. Ltd., by
Adlard & Son, Ltd., 21, *Hart Street, London, W. C.* 1.

COMPLEMENT-FIXATION TESTS IN RELATION TO THE STUDY OF VIRUSES

The earlier literature on this subject contained many conflicting reports, but in subsequent years various improvements in technique introduced by later workers, notably by Bedson, Craigie and others, have led to the more extensive use of complement-fixation tests in the study of viruses.

Analogous to bacterial complement-fixation phenomena, it has been proved that the antiviral sera are capable of fixing complement in the presence of their homologous antigens, just as do antibacterial sera with suspensions of the appropriate micro-organism. Such tests are being extensively employed at the present time, and it is probable that they may provide a means by which different viruses can be identified and strains of the same agent more closely studied. Positive complement-fixation tests have been described in many different virus diseases by numerous workers. In vaccinia and variola, the reaction has been studied by Jobling (1906), Sugai (1909), Hammerschmidt and Konschegg (1917), Gordon (1925), Ritossa (1925), Sobernheim (1925, 1927), Gildemeister and Heuer (1928), Bedson and Bland (1929), Gilmore (1931), and Craigie and Wishart (1936); in herpes febrilis, by Takaki, Bonis, and Koref (1926), Bedson and Bland (1929), and Bedson (1931); in herpes zoster, by Bedson and Bland (1929); in psittacosis, by Bedson (1933, 1937); in lymphogranuloma inguinale, by Hecht (1935), and Hildebrand (1936); in human influenza, by Fairbrother and Hoyle (1937), Hoyle and Fairbrother (1937), and Francis, Magill, Rickard, and Beck (1937). In certain neurotropic virus infections of man, specific complement-fixation reactions have been recommended as an aid to diagnosis, and Takaki, Bonis, and Koref (1926) employed them for the differentiation and identification of rabies, Japanese encephalitis, and certain other virus infections of the central nervous system, while, more recently, Howitt (1937) claimed to have differentiated three strains of equine encephalomyelitis virus and that of lymphocytic choriomeningitis by this procedure.

Complement-fixation tests have also been described in certain animal virus diseases, e.g. foot-and-mouth disease by Ciuca (1929); in Shope's fibroma of rabbits (van Rooyen, 1938; Andrewes and Ahlström, 1938); also in infectious myxomatosis of rabbits (van Rooyen and Rhodes, 1938).

The technique of performing a complement-fixation test, using a virus suspension as antigen and its homologous antiserum, is little different from that of carrying out any routine complement-fixation test (see Mackie and McCartney, 1938; Browning and Mackie, 1937). The main difference lies in the fact that, since viruses cannot be grown in large amounts on culture media like ordinary bacteria, antigen consisting of infected tissues must be employed instead. The use of the latter presents two disadvantages, the first of which is that it necessitates the use of an additional antigen and serum control. The second drawback is that since the concentration of virus in the tissues may be relatively low, its complement-binding potency may be correspondingly feeble, so that a finely balanced test must be set-up and sufficient time must be allowed for union of antigen and antibody to take place (see Bedson, 1928).

The most successful of these methods is that used by Bedson and Bland (1929) who allow complement-fixation to occur slowly over a period of hours at a low temperature, similar to the procedure at one time used for increasing the delicacy of the Wassermann test.

The modes of preparing antigen have also received attention, different methods being used according to circumstances. These are referred to below under their respective diseases. The haemolytic system employed is similar to that used for the Wassermann test, and has usually consisted of a 3 or 5 per cent. suspension of either sheep or ox erythrocytes sensitized with about 5 m.h.d. of homologous immune body. Guinea-pig complement is universally employed, and this can be used either fresh, or preserved by Sonnenschein's (1930) method (see Mackie and McCartney, 1938), or else desiccated complement (Craigie, 1931) may be substituted. It is well to use the mixed serum of several animals. The minimum haemolytic dose of complement is determined in the usual way by titrating it on sensitized corpuscles to be used for the particular experiment, and the test can be performed by the addition of a fixed volume of serum-antigen mixture to varying amounts of complement, or vice versa. Thus, certain workers have preferred to use a fixed amount of complement in their tests, usually about 4 m.h.d., and add varying dilutions of serum, whereas others have chosen to employ a fixed volume of serum with increasing quantities of complement, ranging from 4 to 10 m.h.d. Others have diluted the antigen and kept the remaining reagents constant in volume (Craigie and Wishart, 1936).

Presentation of results.

One may present the result in terms of the dilution which gives positive fixation with the particular antigen in the presence of a constant amount of complement. It may also be reported according to the number of m.h.d. of complement fixed by a constant mixture of antigen and serum. It must be emphasized that unless the same pooled complement is used throughout a series of tests comparisons cannot be made, since the deviability of different specimens of complement may vary considerably (Noguchi and Bronfenbrenner, 1911). Either method is equally good, but many workers, including ourselves, have preferred to use a fixed quantity of serum, with four or five increasing amounts of complement such as 2, 4, 6, 8, and 10 m.h.d. respectively. The third method is described later.

Time and temperature.

The sequence in which the reagents are added conforms to standard serological practice, antigen, serum, and complement being added in that order. These are incubated together for either 1 hour at 37° C. as advised by Ciuca (1929) and Laidlaw and Dunkin (1931), or else for 18 to 20 hours at 8° to 10° C. as advocated by Bedson (1928), Bedson and Bland (1929), and Craigie and Wishart (1936). Others, such as Hoyle and Fairbrother (1937), in their tests with influenza virus, incubate the mixtures for 4 hours at 37° C., followed by a further period of 24 hours at 0° C., as these workers have pointed out that since the fixation of complement by mixtures of antigen and antibody may be slow, prolonged interaction is necessary. Owing to the fact that complement deteriorates rapidly if maintained at 37° C. for longer than 2 to 3 hours, when fixation is performed over a period of 16 to 18 hours' duration, low temperature is obligatory (see also Thomson, Hazen, and Buchbinder, 1932). Thus, in a review of the literature dealing with this subject, Parker and Muckenfuss (1933) have suggested that the failure of the earlier investigators to demonstrate specific complement-fixation with variola-vaccinia virus, can be attributed to the fact that the virus and antigen were incubated for too short a time.

Technique.

Craigie and Wishart (1936) recommend the following scheme for performing the variola-vaccinia complement-fixation test in which they dilute the antigen, the preparation of which is described on p. 88.

Scheme for the Variola-Vaccinia Complement-Fixation Test

TABLE V

Test.

(*a*) Antigen, serial dilutions . . . . . 0·2 c.c.
(*b*) Complement, 3 m.h.d. in 0·2 c.c. . . . . 0·2 c.c.
(*c*) Serum, constant dilution . . . . . 0·1 c.c.

Controls.

(1) Antigen, serial dilutions as in (*a*) . . . . . 0·2 c.c.
Complement, 1·5 m.h.d. in 0·2 c.c. . . . . 0·2 c.c.
Saline . . . . . 0·1 c.c.
(2) Complement, 3 m.h.d. in 0·2 c.c. (diluted 1 in 3) distributed as follows, the object of the arrangement being to ascertain whether complement had deteriorated during the long period of 16 to 18 hours' fixation at low temperature:

Tube	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
Diluted comp. 1 in 3 . .	0·4	0·3	0·2	0·15	0·4	0·3	0·2	0·15	c.c.
Saline	0·1	0·2	0·3	0·35	..	0·1	0·2	0·25	c.c.
Serum, constant dil. as in (*c*) .	..	..	..	..	0·1	0·1	0·1	0·1	c.c.
Estimated m.h.d. of complement present . . .	2·0	1·5	1·0	0·75	2·0	1·5	1·0	0·75	m.h.d.

Test and controls kept in cold room overnight 16 to 18 hours; then after standing at room temperature for 30 minutes, the haemolytic system is added. This consists of a 5 per cent. suspension of washed sheep cells sensitized with 5 minimum sensitizing doses of amboceptor. 0·25 c.c. of the cell suspension is added to each tube. The tests are read after incubation for 30 minutes at 37° C.

The complement employed for the reaction consists of guinea-pig serum which has been dried from the frozen state (see Craigie, 1931). Since prolonged cold-room fixation is necessary, it will be observed from Table V that a special control is included, in order to ascertain whether the different dilutions of complement employed have deteriorated during the period of 16 to 18 hours' fixation.

The serum employed is antivaccinial serum derived from rabbits immunized against vaccinia virus, and the antigen consists of a saline extract of variolous material prepared according to the procedure described below.

ANTIGENS USED, WITH NOTES ON THE CONDUCT OF INDIVIDUAL TESTS

Many different kinds of preparation have been employed. Bedson and Bland (1929) used a 5 per cent. suspension of tissue containing either vaccinia or herpes virus made up in M/50 phosphate buffer solution of pH 7·6 and clarified by slight centrifugation or prolonged standing. A similar 5 per cent. suspension of virulent mouse spleen containing psittacosis virus was found satisfactory for complement-fixation tests in connexion with this virus by Bedson (1933).

Gilmore (1931) used vaccinia virus cultivated for 17 to 24 passages in a medium consisting of a 3 per cent. suspension of rabbit kidney, 30 per cent. rabbit serum, and 67 per cent. Tyrode's solution (see Maitland and Maitland, 1928; and Maitland and Laing, 1930). Cultures were incubated at 37° C. for 4 to 7 days, ground with sand, emulsified in saline, the large

particles allowed to settle, and a 2 per cent. suspension of the upper layer made in saline.

Vaccine calf lymph was shown to fix complement in the presence of sera derived from vaccinated calves by Jobling (1906); this same antigen has been employed for tests with anti-smallpox sera with positive results by Dahm (1909) and Kolmer (1916); and further work on the subject has been recorded by Xylander (1909), Bermbach (1909), Teissier and Gastinel (1912), and Konschegg (1915). Boiled vaccine lymph was also found to behave as an equally effective antigen in complement-fixation tests with vaccinial or variolar antiserum (Lurje and Wolkowitsch, 1928; Glaser and Koref, 1928).

Wollman and Urbain (1928) demonstrated positive complement-fixation when using an emulsion of brain tissue infected with vaccinia virus and guinea-pig immune serum. Myers and Chapman (1937) used as antigen vaccinia virus grown in the developing chicken embryo.

The vesicle fluid derived from cases of herpes zoster has been shown to possess complement-fixing properties with convalescent serum derived from patients recovered from the disease by Netter, Urbain, and Weissmann-Netter (1924) and by Bedson and Bland (1929). On the other hand, convalescent sera from cases of varicella contained, according to Netter and Urbain (1924), no complement-fixing antibodies for zoster antigen.

Washed suspensions of vaccinial elementary bodies, as well as Seitz filtrates of tissue extracts (which contained the specific precipitable substance of vaccinia), also behaved as suitable antigens for the complement-fixation test (Craigie and Wishart, 1934 *b*).

Dried skin crusts or vesicle or pustule fluid obtained from suspected cases of smallpox have been utilized by Craigie and Wishart (1936) as antigen for the serological diagnosis of smallpox, by the complement-fixation reaction (see p. 87). Desiccated specimens were macerated for a few hours in 8·5 per cent. sodium chloride, and in the case of dried fluid or swabs only 0·2 c.c. of saline was added. After several hours at room temperature 1·8 c.c. of neutral distilled water was added, the suspension centrifuged at high speed for 20 minutes, and the supernatant fluid used. With crusts, 40 mg. was macerated in 0·2 c.c. of 8·5 per cent. sodium chloride, broken-up with a glass rod, 1·8 c.c. of distilled water added, the suspension centrifuged, the supernatant fluid removed and used as antigen in the test.

The sera employed for the reaction have consisted of antivaccinial hyperimmune sera prepared by the immunization of rabbits with either washed elementary bodies or with Seitz filtrates of extracts of lesions.

Parker and Muckenfuss (1933) attempted to prepare a chemically pure antigen by treating infected tissue extracts with ammonium sulphate and carbon dioxide followed by dialysis in collodion sacs. Elution in alkaline solution followed by absorption with kaolin was tried also, but without success, as the antigenic properties of the final product were diminished and became anticomplementary. Alcoholic extracts prepared from variolar crusts have been found satisfactory by Ritossa (1925), but tissue extracts prepared from post-mortem material derived from cases of variola were unsuitable as antigen for complement-fixation tests (see Dahm, 1909; Kryloff, 1911; Konschegg, 1915; Hallenberger, 1917, 1918).

For tests in foot-and-mouth disease, antigen consisting of Berkefeld filtrates prepared from infective tissues was used to fix complement in the presence of serum derived from hyperimmunized cattle (Lourens, 1909).

Unfiltered vesicle fluid also proved to be an equally efficient antigen, and Mezincescu, Baroni, and Calinescu (1923) and also Urbain (1927) used such material in their experiments. Ciuca (1929) has reinvestigated the subject and adduced additional evidence regarding the complement-fixation test in foot-and-mouth disease. The antigen employed by him consisted of either (*a*) an alcoholic extract prepared after Bordet's method, from vesicle epithelium or the feet of infected guinea-pigs, diluted 1:10 or 1:50 before use; or (*b*) vesicle fluid derived from lesions on the feet of guinea-pigs 24 hours after intradermal inoculation, diluted 1:10 or 1:50 in phosphate solution of pH 7·6, centrifuged, passed through a Seitz filter, stored for 15 to 60 days in a cold room, and then used for test; or (*c*) a watery suspension prepared from the foot lesions of guinea-pigs excised 18 to 24 hours after inoculation, allowed to macerate for 15 days, filtered through a Seitz disk, and used as antigen.

With these antigens the presence of complement-fixing antibodies was demonstrated in the sera of guinea-pigs 7 days after infection with virus; they attained a maximum on the twenty-first day, lasted 55 days, and disappeared after 72 days.

Ciuca (1929) maintained that the reaction was highly specific, as non-specific complement-fixation was never produced by normal guinea-pig serum. Furthermore, it was possible to identify and differentiate strains of virus such as the Vallée O, Vallée A, and Waldmann C types by the specificity of their reactions.

Influenza

Saline tissue extracts obtained from mouse lung infected with influenza virus have been used as antigen by Fairbrother and Hoyle (1937) and Hoyle and Fairbrother (1937). The antigenic fraction of such extracts appeared to be soluble and could not be deposited by centrifugation at 13,000 r.p.m. for 45 minutes. Its activity was destroyed by heating at 70° C. for 15 minutes, or by adding N/5 sodium hydroxide for 15 minutes, and was considerably reduced by treatment with N/5 hydrochloric acid. It was partially precipitated by treating mouse lung suspensions with 1 per cent. acetic acid and could be recovered by redissolving the deposit in buffer solution of pH 7·3. Hoyle and Fairbrother (1937) state that this complement-fixing antigen which they have prepared from influenza virus in mouse lung is a soluble substance probably liberated during multiplication of the virus in the tissues.

Lymphogranuloma Inguinale

In lymphogranuloma inguinale (L.G.I.), Hecht (1935) and Hildebrand (1936) have described complement-fixation tests, and the latter obtained specific fixation using immune rabbit serum with a variety of different antigens such as extracts prepared from infected mouse brain, *M. rhesus* monkey brain, guinea-pig lymphatic glands, or human buboes affected with the disease. Tests with patients' sera proved disappointing, for only partial fixation resulted between them and antigen prepared from human buboes, while no precipitation occurred with infected guinea-pig lymphatic gland extract.

Rift Valley Fever

Broom and Findlay (1932) have demonstrated the existence of specific complement-fixing antibodies in the sera of humans and animals recovered from the disease. The antigen used was either a 2 per cent. saline extract prepared from fresh infected mouse or rat liver, or from similar tissues

which had been desiccated over phosphorous pentoxide. For the test, three different dilutions of the antigen suspension were used, namely, 0·25 c.c. of 2 per cent., 1 per cent., and 0·5 per cent. antigen suspensions, respectively, to which 0·25 c.c. containing 3 m.h.d. of complement and 0·25 c.c. of immune serum was added. The mixtures were incubated at 37° C. for 1 hour; 0·25 c.c. of a 2·5 per cent. suspension of sheep erythrocytes sensitized with 5 m.h.d. of amboceptor was then added; the incubation was continued for a further 10 minutes and the results then read. The serum control consisted of normal human or animal serum, the antigen control being normal mouse or rat liver. In addition, an extra control was included in which a known positive serum was tested against infected liver tissue as well as normal liver.

Dengue

Partial success with complement-fixation reactions has been claimed by Simmons, St. John, and Reynolds (1931) who at first tried to perform the test with antigen consisting of infected human blood, or the liver or spleen of infected *Macacus philippinensis* monkeys, but with negative results. Later, however, they were able to demonstrate fixation with antigen consisting of ground-up infected aëdes mosquitoes, for which purpose 279 insects were suspended in 95 per cent. alcohol, allowed to stand for 12 days, centrifuged, and the supernatant fluid diluted to make a 1:10 suspension in physiological saline.

With such antigen Simmons and his co-workers were able to show the presence of specific complement-fixing antibodies in the sera of individuals who had suffered from dengue fever, the reaction being found positive from the first to the ninth day of the patient's illness.

Yellow Fever

Frobisher (1929) and Davis (1931) have demonstrated that the sera of persons or monkeys after recovery from an attack of yellow fever develop specific complement-fixing antibodies. An antigen suitable for the test has been prepared by Frobisher (1931 *a*) according to the following procedure: The livers of several rhesus monkeys which had died from yellow fever were excised, freed from adventitious tissue, weighed, cut into small fragments with scissors, and ground in a mortar with sand to form a paste. This was then spread over the surface of a flat dish, placed in a desiccator over sulphuric acid, dried *in vacuo* for 18 to 24 hours, and the desiccate placed in hermetically sealed containers and stored in a refrigerator until required. Although saline suspensions of such material were found to act as an efficient antigen, better results were obtained using dried liver tissue from which the fat had been removed. This was done by placing the material in a Soxhlet apparatus and extracting the fat by treating it with ether for 8 hours or longer. The antigen was now prepared from the fat-free liver substance by first treating it with hypertonic salt solution and then diluting the mixture before use. To a measured bulk of tissue, about one-half of its weight of a 9 per cent. solution of sodium chloride was added, allowed to macerate for 4 to 24 hours in the refrigerator, diluted to 0·9 per cent. sodium chloride by addition of distilled water, centrifuged for 30 minutes, and the supernatant fluid passed through a Berkefeld V filter, this filtrate being used as antigen for the test.

Technique. Frobisher (1931) employed the following method: Sera to be tested were inactivated by heating at 56° C. for 30 minutes; 0·2 c.c. was placed in one test-tube, and 0·1 c.c. plus 0·1 c.c. of saline added

to another; followed by the addition of 0·2 c.c. of diluted antigen to each tube. In a third tube 0·2 c.c. of physiological saline was placed. To all three tubes 2 m.h.d. of guinea-pig complement were added. The mixtures were then incubated at 37° C. for 1 hour, at the end of which 0·4 c.c. of a haemolytic system consisting of 5 per cent. sheep erythrocytes sensitized with 2 units of sheep antibody was added. The test was replaced in the incubator at 37° C. for a further period of 30 minutes, and the result read at the end of this time or at such time as the controls showed complete lysis.

Psittacosis

The complement-fixation reaction has been proved a valuable aid to the diagnosis of suspected cases of this disease (Bedson, 1935). The antigen employed for the reaction consists of a 5 per cent. saline suspension of virulent mouse spleen, which has been allowed to stand in a refrigerator at +4° C. for one week, in order to annul any traces of natural complement (or alternatively, it is heated at 56° C. for 30 minutes), and 0·1 c.c. bulk used for the test. Boiled antigen was also shown to be effective by Bedson (1937). The patient's serum to be examined is diluted 1 : 1 to 1 : 8 and 0·1 c.c. of each dilution employed. Two m.h.d. of complement are used throughout the test. The mixtures are incubated at room temperature for 2 to 3 hours, 0·2 c.c. of sensitized cells added, the tubes placed in the water-bath at 37° C. for 30 minutes, and the reaction read at the end of that time. Bedson points out that the specific complement-fixing power of these human psittacosis sera is of a low order and that a fairly balanced test is essential to elicit the reaction. Rigorous controls are also necessary to eliminate false reactions. The reaction has been found to be positive as early as the twelfth day and as late as the fifth week of the patient's illness. How long complement-fixing antibodies remain in the patient's serum has not yet been determined.

TESTS WITH BOILED ANTIGENS

'Koktoantigen' or boiled infected brain tissue was used as antigen for the complement-fixation tests by Torikata (1917), Nakagawa (1924), and Takaki, Bonis, and Koref (1926). Their method was as follows: Brain tissue infected with a neurotropic virus preserved for 10 days or longer in 50 per cent. glycerine was ground up in 0·85 per cent. sodium chloride to yield a 10 per cent. suspension of brain tissue. This material was boiled for 30 minutes in a water-bath; allowed to stand for 12 to 24 hours, sharply centrifuged, and the supernatant opalescent fluid used as antigen in the tests. With such material Takaki, Bonis, and Koref (1926) successfully identified and differentiated by specific complement-fixation certain neurotropic viruses, such as those of Japanese and European encephalitis (Levaditi). Their tests also revealed the identity of the last-named virus with that of herpes febrilis (Doerr, 1926; Luger and Lauda, 1925). This work also established that the viruses of Silberstein (1925), rabies, Koritschoner, and neurovaccine (Levaditi) were separate and unrelated entities.

Gildemeister and Heuer (1928) have criticized the findings of Takaki, Bonis, and Koref (1926) for, although the former agreed that complement-fixation occurred between antivaccinial serum and homologous brain 'koktoantigen', they questioned the specificity of the reaction as claimed by Takaki and his colleagues in respect of herpes virus. Gildemeister and Heuer pointed out that normal brain tissue occasionally produced non-specific complement-fixation and, moreover, owing to the unreliability of

such tests when performed on experimental animals, they expressed doubts concerning its practical value as a diagnostic reaction in man.

The identification of certain neurotropic viruses by the results of complement-fixation tests have also received the attention of Howitt (1937). This author employed infected desiccated brain tissue which was ground up with ether; stored for some hours on ice; the ether removed; the residue suspended in 0·85 per cent. saline; left for 6 to 12 hours on ice; repeatedly frozen and thawed at 37° C.; centrifuged; the supernatant fluid removed and used as antigen. The antigen was not heated and the tests were carried out by allowing complement-fixation to occur over a period of 16 hours at refrigerator temperature. Use was made of this technique for investigating the immunological cross-reactions of the Eastern American, Western American, and Moscow No. 2 strains of equine encephalomyelitis virus as well as the virus of lymphocytic choriomeningitis. The immune serum employed was derived from guinea-pigs hyperimmunized against individual strains, and the results showed that each of the three different strains of equine encephalomyelitis virus investigated could by the procedure in question be differentiated from each other, as well as from the virus of benign lymphocytic choriomeningitis.

The complement-fixation test has also been performed in psittacosis with boiled antigen by Bedson (1937). Material was prepared as follows: a heavily infected mouse spleen was suspended in 5·0 c.c. of M/50 phosphate buffer solution of pH 7·6, and allowed to sediment for 24 hours in the refrigerator; the supernatant fluid was removed, and centrifuged for 1½ hours on the angle centrifuge; the supernatant fluid was discarded, and the deposited material resuspended in 5·0 c.c. of phosphate buffer, following which coarse fragments were removed by light centrifugation; the suspension was finally steamed for 30 minutes and employed as antigen in the test. The control antigen consisted of normal mouse spleen which was similarly treated.

Boiled antigen prepared by this method was found useful for demonstrating the presence of complement-fixing antibodies in the sera of human cases of psittacosis, and Bedson found that heating not only increased the sensitivity of the material, but also made it easier to preserve and safer to handle.

In the case of herpes virus, however, heated antigen has given disappointing results, and Bedson and Bland (1929) reported that heating greatly diminished the antigenic activity of tissue extracts prepared from infected guinea-pig feet.

THE IMPORTANCE OF ADEQUATE CONTROLS IN COMPLEMENT-FIXATION TESTS APPLIED TO THE STUDY OF VIRUSES

It cannot be too strongly emphasized that all serological tests such as complement-fixation, agglutination, or neutralization tests should be carefully controlled if the results are to be of value. As alluded to earlier in this chapter, the subject demands particular attention in the study of human virus infections, since these agents are only cultivable along with living cells and the presence of the latter may *per se* cause certain non-specific effects. For example, although it is well known that many viruses function as efficient antigens in specific complement-fixation reactions, yet earlier literature on this same subject contained many contradictory reports. In reference to this point, Parker and Muckenfuss (1933) have pointed out that some of the results reported were useless, either because they were insufficiently controlled, or else because they

did not contain sufficient data. For instance, when attempting to prepare a hyperimmune guinea-pig antivaccinial serum by inoculating the animal with vaccinia virus grown in rabbit testis, the experiment should be controlled by preparing another serum derived from a separate guinea-pig which has received a similar number of doses of normal rabbit testicular extract. The serum of a normal guinea-pig, if employed as a control under such circumstances, would obviously be an inadequate check. This control is essential, for although the resulting antivaccinial serum may show true complement-fixation effects, it is important to guard against fallacious non-specific fixation caused by inoculation of a guinea-pig with rabbit protein. An additional antigen control consisting of normal healthy testis tissue should also be incorporated in the experiment in every case.

The state of our knowledge regarding the extent to which the parenteral injection of tissues derived from one species of animal into those of another gives rise to various immunological effects is as yet incompletely understood; for example, in the case of the Forssman reaction, the inoculation of a rabbit with the kidney tissue of a guinea-pig leads to the production of an haemolysin in the rabbit's serum for sheep erythrocytes. A hypothetical instance in which this phenomenon would interfere with the performance of a complement-fixation test applied to a virus disease would be as follows: if a rabbit were immunized with a virus contained in an infected guinea-pig's kidney, the rabbit's serum would not only develop complement-fixing antibodies for the particular agent introduced, but would simultaneously produce an haemolysin for sheep cells. Under such circumstances an haemolytic system consisting of sensitized ox cells would have to be substituted for sheep cells. During the course of serological studies in connexion with viruses, a careful watch should always be kept for the appearance of phenomena of a similar type, and no positive result should be accepted unless it has been proved beyond doubt that the effect is specifically due to the antigenic properties of the virus itself, and not one peculiar to the tissues of the animal in which the virus has been cultivated.

In their carefully controlled experiments on influenza virus, Fairbrother and Hoyle (1937) have revealed the practical importance of this point, for, when they endeavoured to apply the complement-fixation test to ferret sera, they found from their control experiments that the complement-fixation which occurred between anti-influenzal ferret serum and influenzal mouse lung was non-specific in character, as the same effect was obtained with normal mouse lung as antigen. Likewise the work of Gildemeister and Heuer (1928) showed that normal rabbit brain tissue could behave in a similar manner, and Hildebrand (1936) also disclosed that rabbit serum and normal human lymphatic glands could act in the same way. In the past five years the researches of Bedson, Craigie, Fairbrother, and others, have improved the technique of complement-fixation reactions applied to the study of viruses, and their results have indicated the great possibilities offered by such tests in differentiating and identifying viruses.

The principal obstacle offered to the future development of this work, along lines which may be of practical utility in human diagnosis, still remains that of non-specific complement-fixation caused by tissue proteins from which viruses are inseparable. As an academic problem the subject of non-specific complement-fixation is a complex one, and this has been intensively investigated by Mackie and Finkelstein (1928) who have made a special study of non-specific complement-fixation produced by the

interaction of normal serum and certain non-antigenic substances. Mackie and Finkelstein's work has also drawn attention to the possibility of increasing the amount of complement fixed by the addition of cholesterol to the antigen. Gilmore (1931) endeavoured to utilize this principle in order to enhance the amount of complement fixed in tests with anti-vaccinial sera, but the results were not satisfactory. Later, Mackie and Finkelstein (1930) investigated the phenomenon of complement-fixation produced by the interaction of normal serum with bacterial suspensions, and from their work they found that the normal sera of various mammals (e.g. man, ox, sheep, horse, pig, white rat, rabbit, and guinea-pig) possessed the property of fixing complement with a wide variety of different bacteria.

These observations are of some interest because previously, Schultz, Bullock, and Lawrence (1928) suggested that positive complement-fixation, using antivaccinial serum and vaccinial antigen prepared from infected rabbit skin, was in reality a non-specific effect attributable to the existence of contaminant bacteria occurring in the antigen. Schultz's allegations were subsequently reinvestigated by other workers, but received no support (see Craigie and Tulloch, 1931).

In conclusion, we should like to add that, although specific complement-fixing antibodies have been demonstrated in a great many virus diseases, these reactions are as a whole so delicate that the execution of such tests, their adequate control, and interpretation of results demand the utmost care and attention.

REFERENCES

Amies, C. R. (1934), *Brit. J. exp. Path.*, **15**, 314.
Andrewes, C. H. (1928), *J. Path. Bact.*, **31**, 671.
Andrewes, C. H., and Ahlström, C. G. (1938), *J. Path. Bact.*, **47**, 87.
Andrewes, C. H., Laidlaw, P. P., and Smith, W. (1935), *Brit. J. exp. Path.*, **16**, 566.
Bedson, S. P. (1928), *Brit. J. exp. Path.*, **9**, 235.
—— (1931), *Brit. J. exp. Path.*, **12**, 254.
—— (1932), *Brit. J. exp. Path.*, **13**, 65.
—— (1933), *Brit. J. exp. Path.*, **14**, 162.
—— (1935), *Lancet*, **2**, 1277.
—— (1937), *Lancet*, **2**, 1477.
—— (1938), *Brit. J. exp. Path.*, **19**, 353.
Bedson, S. P., and Bland, J. O. W. (1929), *Brit. J. exp. Path.*, **10**, 393.
Bermbach, P. (1909), *Zbl. Bakt.*, Abt. I, orig., **49**, 618.
Broom, J. C., and Findlay, G. M. (1932), *Lancet*, **1**, 609.
Browning, C. H., and Mackie, T. J. (1937), Muir and Ritchie's *Text-book of Bacteriology*, Oxford University Press, London.
Burnet, F. M. (1937), *Med. res. Counc. spec. Rep.*, no. 220, H.M .Stationery Office, London.
Burnet, F. M., Keogh, E. V., and Lush, D. (1937), *Aust. J. exp. Biol. med. Sci.*, **15**, 231.
Ciuca, A. (1929), *J. Hyg., Camb.*, **28**, 325.
Clark, W. M. (1928), *The Determination of Hydrogen Ions*, 3rd ed., pp. 209, 211, Williams & Wilkins, Baltimore.
Craigie, J. (1931), *Brit. J. exp. Path.*, **12**, 75.
—— (1932), *Brit. J. exp. Path.*, **13**, 259.
Craigie, J., and Tulloch, W. J. (1931), *Med. res. Counc. spec. Rep.*, no. 156, H.M. Stationery Office, London.
Craigie, J., and Wishart, F. O. (1934*a*), *Brit. J. exp. Path.*, **15**, 390.
—— —— (1934*b*), *Trans. roy. Soc. Can.*, **28**, sect. V, 91.
—— —— (1935), *Trans. roy. Soc. Can.*, **29**, sect. V, 57.
—— —— (1936), *Canad. publ. Hlth. J.*, **27**, 371.
—— —— (1936*a*), *J. exp. Med.*, **64**, 819.
—— —— (1936*b*), *J. exp. Med.*, **64**, 803.
Dahm, (1909), *Zbl. Bakt.*, Abt. I, orig., **51**, 136.
Davis, G. E. (1931), *Amer. J. Hyg.*, **13**, 79.
Doeer, R. (1926), *Zbl. Bakt.*, Abt. I, orig., **97**, 76.*
Douglas, S. R., Smith, W., and Price, L. R. W. (1929), *J. Path. Bact.*, **32**, 99.

FAIRBROTHER, R. W. (1930), *Brit. J. exp. Path.*, **11**, 43.
FAIRBROTHER, R. W., and HOYLE, L. (1937), *J. Path. Bact.*, **44**, 213.
FAIRBROTHER, R. W., and HURST, E. W. (1930), *J. Path. Bact.*, **33**, 17.
FINDLAY, G. M. (1931), *Brit. J. exp. Path.*, **12**, 9.
—— (1936), *Brit. J. exp. Path.*, **17**, 89.
FRANCIS, T., and MAGILL, T. P. (1935), *J. exp. Med.*, **62**, 433.
FRANCIS, T., MAGILL, T. P., RICKARD, E. R., and BECK, M.D. (1937), *Amer. J. publ. Hlth.*, **27**, 1141.
FROBISHER, M. (1929), *Proc. Soc. exp. Biol., N.Y.*, **26**, 846.
—— (1931), *Amer. J. Hyg.*, **13**, 585.
—— (1931 *a*), *Amer. J. Hyg.*, **14**, 147.
GILDEMEISTER, E., and HEUER, G. (1928), *Zbl. Bakt.*, Abt. I, orig., **105**, 326.
GILMORE, E. ST. G. (1931), *Brit. J. exp. Path.*, **12**, 165.
GLASER, F., and KOREF, O. (1928), *Z. ImmunForsch.*, **56**, 88.
GORDON, M. H. (1925), *Med. res. Counc. spec. Rep.*, no. 98, H.M. Stationery Office, London.
—— (1935), *Lancet*, **1**, 1527.
—— (1935 *a*), *Lancet*, **2**, 104.
GOYAL, R. K. (1935), *J. Immunol.*, **29**, 111.
HALLENBERGER, O. (1917), *Berl. klin. Wschr.*, **54**, 543.
—— (1917), *Münch. med. Wschr.*, **64**, 985.
—— (1918), *Z. klin. Med.*, **86**, 358.
HAMMERSCHMIDT, J., and KONSCHEGG, A. (1917), *Münch. med. Wschr.*, **64**, 871.
HASSKÓ, A., VÁMOS, L., and THOROCZKAY, M. (1938), *Z. ImmunForsch.*, **93**, 80.
HECHT, H. (1935), *Wien. klin. Wschr.*, **48**, 1389.
HILDEBRAND, H. (1936), *Zbl. Bakt.*, Abt. I, orig., **137**, 245.
HOWITT, B. F. (1937), *Proc. Soc. exp. Biol., N.Y.*, **35**, 526.
HOYLE, L., and FAIRBROTHER, R. W. (1937), *J. Hyg., Camb.*, **37**, 512.
HUGHES, T. P. (Jr.) (1933), *J. Immunol.*, **25**, 275.
HUGHES, T. P., PARKER, R. F., and RIVERS, T. M. (1935), *J. exp. Med.*, **62**, 349.
JENSEN, C. (1935), *Proc. roy. Soc. Med.*, **28**, 1007.
JOBLING, J. W. (1906), *J. exp. Med.*, **8**, 707.
KOLMER, J. A. (1916), *J. Immunol.*, **1**, 59.
KOLMER, J. A., and RULE, A. M. (1935), *J. Immunol.*, **29**, 175.
KONSCHEGG, A. (1915), *Münch. med. Wschr.*, **62**, 4.
—— (1915), *Wien. klin. Wschr.*, **28**, 442.
—— (1918), *Dtsch. med. Wschr.*, **44**, 9.
KRYLOFF, D. (1911), *Zbl. Bakt.*, Abt. I, Orig., **60**, 651.
LAIDLAW, P. P., SMITH, W., ANDREWES, C. H., and DUNKIN, G. W. (1935), *Brit. J. exp. Path.*, **16**, 275.
LEDINGHAM, J. C. G. (1935), *Lancet*, **2**, 45.
—— (1937), *Brit. J. exp. Path.*, **18**, 436.
LEDINGHAM, J. C. G., MORGAN, W. T. J., and PETRIE, G. F. (1931), *Brit. J. exp. Path.*, **12**, 357.
LOURENS, L. (1909), *IX Int. vet. Congr.*, Hague, p. 3.
LUGER and LAUDA (1925), *Wien. klin. Wschr.*: see TAKAKI *et al.* (1926).
LURJE, M., and WOLKOWITSCH, M. (1928), *Z. ImmunForsch.*, **57**, 140.
MACKENZIE, R. D. (1935), *J. Path. Bact.*, **40**, 65.
MACKIE, T. J., and FINKELSTEIN, M. H. (1928), *J. Hyg., Camb.*, **28**, 172.
—— —— (1930), *J. Hyg., Camb.*, **30**, 1.
MACKIE, T. J., and MCCARTNEY, J. E. (1938), *A Handbook of Practical Bacteriology*, Livingstone, Edinburgh.
MADSEN, T., and JENSEN, C. (1936), *Quart. Bull. Hlth. Org. L. o. N.*, **5**, 708.
MADSEN, T., JENSEN, C., and EAGLES, G. H. (1936), *Quart. Bull. Hlth. Org. L. o. N.*, **5**, 710.
MAGILL, T. P., and FRANCIS, T. (1938), *Brit. J. exp. Path.*, **19**, 273.
MAITLAND, H. B., and LAING, A. W. (1930), *Brit. J. exp. Path.*, **11**, 119.
MAITLAND, H. B., and MAITLAND, M. C. (1928), *Lancet*, **2**, 596.
MEZINCESCU, O., BARONI, V., and CALINESCU, I. (1923), *Ann. Inst. Pasteur*, **37**, 1057.
NAKAGAWA, T. (1924), *Z. ImmunForsch.*, **39**, 173.
NETTER, A., URBAIN, A., and WEISMANN-NETTER (1924), *C.R. Soc. Biol., Paris*, **90**, 75.
NOGUCHI, H., and BRONFENBRENNER, J. (1911), *J. exp. Med.*, **13**, 45.
PARKER, R. F., and MUCKENFUSS, R. S. (1933), *J. infect. Dis.*, **53**, 44.
PARKER, R. F., and RIVERS, T. M. (1935), *J. exp. Med.*, **62**, 65.
—— —— (1936), *J. exp. Med.*, **63**, 69.
—— —— (1937), *J. exp. Med.*, **65**, 243.
PERDRAU, J. R., and TODD, C. (1933), *Proc. roy. Soc.*, B, **112**, 288.
RITOSSA, P. (1925), *Pediatria*, **33**, 917.

RIVERS, T. M., and WARD, S. M. (1937), *J. exp. Med.*, **66**, 1.
SCHULTZ, E. W., BULLOCK, L. T., and LAWRENCE, F. (1928), *J. Immunol.*, **15**, 243.
SILBERSTEIN, F. (1925), *Z. ges. exp. Med.*: see TAKAKI *et al.* (1926).
SIMMONS, J. S., ST. JOHN, J. H., and REYNOLDS, F. H. K. (1931), *Philipp. J. Sci.*, **44**, 1.
SMITH, W. (1930), *J. Path. Bact.*, **33**, 273.
SMITH, W., and ANDREWES, C. H. (1938), *Brit. J. exp. Path.*, **19**, 293.
SMITH, W., ANDREWES, C. H., and LAIDLAW, P. P. (1935), *Brit. J. exp. Path.*, **16**, 291.
SOBERNHEIM, G. (1925), *Ergebn. Hyg. Bakt.*, **7**, 133.
—— (1927), *Handbuch der Pockenbekämpfung und Impfung*, Herausgegeben von Lentz und Gins, R. Schoetz, Berlin.
SONNENSCHEIN, C. (1930), *Z. Immunforsch*, **67**, 512.
SUGAI, T. (1909), *Zbl. Bakt.*, Abt. I, orig., **49**, 650.
TAKAKI, J., BONIS, A., and KOREF, O. (1926), *Z. ImmunForsch.*, **47**, 431.
TANAKA, K. (1902), *Zbl. Bakt.*, Abt. I, orig., **32**, 726.
TEISSIER, P., and GASTINEL, P. (1912), *C.R. Soc. Biol., Paris*, **73**, 264.
—— —— (1912), *C.R. Acad. Sci., Paris*, **155**, 1170.
THOMSON, R., HAZEN, E. L., and BUCHBINDER, L. (1932), *J. Immunol.*, **22**, 189.
TORIKATA, R. (1917), *Koktopräzipitinogene und Koktoimmunogene*, Bern: quoted by Nakagawa (1924).
URBAIN, A. (1927), *Traitè. Édition de la Revue de Pathologie comparée et d'Hygiène générale*, p. 174, Paris.
VAN ROOYEN, C. E. (1938), *Brit. J. exp. Path.*, **19**, 156.
VAN ROOYEN, C. E., and RHODES, A. J. (1938), *Zbl. Bakt.*, Abt. I, orig., **142**, 149.
WISHART, F. O., and CRAIGIE, J. (1936), *J. exp. Med.*, **64**, 831.
WOLLMAN, E., and URBAIN, A. (1928), *C.R. Soc. Biol., Paris*, **99**, 480.
WOOLEY, J. G., and ARMSTRONG, C. (1934), *Publ. Hlth. Rep., Wash.*, **49**, 1495.
WOOLEY, J. G., ARMSTRONG, C., and ONSTOTT, R. H. (1937), *Publ. Hlth. Rep., Wash.*, **52**, 1105.
XYLANDER, (1909), *Zbl. Bakt.*, Abt. I, orig., **51**, 290.

PART IV.

TISSUE CULTIVATION IN THE STUDY OF VIRUS DISEASES.

The following pages contain an account of the most successful methods whereby satisfactory tissue cultures of viruses may be obtained. Work of this type necessitates the help of a team of investigators with the use of elaborate equipment. My work has been performed in collaboration with Dr A. J. Rhodes in an ordinary bacteriological laboratory. A few small contributions to the subject have been made in the form of simplifications of technique. The primary object of this research was to watch the development of inclusion and elementary bodies under the microscope at different stages of growth, and subsequently to transfer these cultures to a micromanipulator and then to microdissect the inclusions which had formed. The technical difficulties encountered in such operations are great, but the results are well worth the trouble expended, as microdissection of virus inclusions supplies information concerning the morphology of inclusions which could never be provided by histological sections. The majority of experiments on these lines were frustrated owing to misfortunes sustained during the handling of tissues, but on one occasion it was possible to obtain a tissue culture of ectromelia virus in tissue culture, and having watched the formation of the inclusion body, subsequently to dissect it under the microscope. The

results/

results were never published as difficulty was encountered in being able to repeat the results, and tissue cultures bearing inclusions suitable for manipulation were difficult to grow.

Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY
Vol. XLV., No. 1, pp. 253-256, 1937

Inclusion Bodies in Corneal Tissue Cultures Infected with Vaccinia Virus

BY

A. J. RHODES AND C. E. VAN ROOYEN

From the Department of Bacteriology, University of Edinburgh

Reprinted from The Journal of Pathology and Bacteriology
Vol. XLV., No. 1, pp. 253-256, 1937

578 . 085 . 23 : 616 . 913—022 . 6

INCLUSION BODIES IN CORNEAL TISSUE CULTURES INFECTED WITH VACCINIA VIRUS.

A. J. Rhodes and C. E. van Rooyen.

From the Department of Bacteriology, University of Edinburgh.

(Plate XLII.)

Of recent years tissue culture has been used increasingly in the study of the filterable viruses and has proved especially suitable for the demonstration of inclusion bodies. Thus Bland and Canti (1935) demonstrated the characteristic life cycle of the psittacosis virus when growing within epithelial and fibroblastic cells of the chick embryo. Working with vaccinia virus Rivers, Haagen and Muckenfuss (1928-29, 1929) showed that Guarnieri bodies appeared in tissue cultures of rabbit corneal epithelium. Goodpasture, Woodruff and Buddingh (1932) found that this virus produced inclusion bodies in chick embryo fibroblasts *in vitro*. Recently Rhodes and van Rooyen (1937) have demonstrated similar inclusion bodies in fibroblasts in vaccinial keratitis of the rabbit. In this paper, work is described which has been carried out in order to study the possible development of Guarnieri or other inclusion bodies in tissue cultures of rabbit cornea infected with vaccinia virus.

Methods.

Vaccinia virus. The strain of virus was the same as that used in our previous work (Rhodes and van Rooyen, 1937), and had been maintained by repeated testicular passage through rabbits. Virus was used in either of two ways : (1) desiccated testis was ground up in 9 volumes of Tyrode's solution, centrifuged slowly and the supernatant used as " desiccated " virus ; (2) a rabbit was injected intratesticularly with the " desiccated " virus and four days later the testis was removed and ground up in Tyrode's solution to yield a 1 : 10 suspension. After centrifuging slowly the supernatant constituted " fresh " virus.

Technique. Two different methods were adopted to grow rabbit corneal cells (Rhodes, 1937). (1) *Slide technique.* Small squares of rabbit cornea, previously immersed in virus for 30 minutes, were placed in the centres of coverslips and surrounded by a few drops of nutrient fluid (chick plasma, rabbit serum, rabbit spleen extract). The coverslips were then picked up with hollow-ground slides, inverted, and incubated at 37° C. Examined microscopically in the living unstained condition, new growths of epithelium could be seen spreading out as sheets around the implants after 24 hours.

Coverslips were removed at daily intervals of from one to five days and in order to search for inclusion bodies the cultures were stained by the following method (Rhodes, 1937). The coverslips were floated face downwards in a watchglassful of hæmatein solution for 10 minutes and then washed in methylated spirit and tap water. The cultures were partially dehydrated with methylated spirit and absolute alcohol before being counterstained with alcoholic eosin for 1 minute. Finally they were washed with absolute alcohol and left in xylol until completely dehydrated. Some hundreds of such cultures were set up, inoculated with either desiccated or fresh virus.

(2) *Tube technique* (modified from Rivers, Haagen and Muckenfuss, 1928-29, 1929). A rabbit's eye was freed in the orbital cavity and the corneal surface cross hatched with a sterile cataract knife dipped in virus. The cornea was then dissected out, cut into 8-12 pieces and immersed in a suspension of virus for 30 minutes. Thereafter an infected piece of cornea was placed horizontally on the surface of a layer of clotted nutrient fluid in a small glass tube and covered over with more fluid. After incubation at 37° C. for varying periods up to 5 days the whole clot was removed, fixed, dehydrated and embedded, and sections were cut parallel to the long axis of the tube. Sections were stained by Mann's method (Ford's modification, 1934) and showed in the centre of the clot a radial section of cornea with superficial epithelium, substantia propria and Descemet's membrane. The advantages of this method are that a perfect histological preparation can be obtained and that the development of cells is as readily followed in the substantia propria as in the superficial epithelium. Approximately 100 such cultures were prepared, using desiccated or fresh virus.

Controls. Over 50 normal cultures, obtained by both methods, were also examined at daily intervals from 1 to 5 days.

Results.

Slide technique. Here the object was to study the possible development of Guarnieri or other inclusion bodies in cultures of epithelial cells. Of some hundreds of infected cultures examined microscopically at varying periods of growth, both in the living condition and after staining, none showed any departure from normal; they grew as rapidly as the controls, with no evidence of degeneration or of inclusion bodies.

Tube technique. Here the object was to study the possible development of inclusions in cultures containing all the corneal layers. Infected cultures of 24 hours' growth showed an increase in the numbers of fibroblasts in the substantia propria. The cytoplasm of many of these fibroblasts contained inclusion bodies in small numbers. They were spherical, brightly eosinophilic granules. By 48 hours the fibroblasts had further increased in number (fig. 1) and now in size also, presenting large oval nuclei, and a cytoplasm containing numerous inclusion bodies (fig. 2). The latter were remarkably uniform in size, the great majority measuring from 0·6 to 0·8 μ in diameter. A few small forms (0·3 μ) were also noted, as well as larger forms measuring 2 μ in diameter.

Specimens were examined daily until the 5th day and continued to show bodies of similar shape, size and staining reaction within

VACCINIAL INCLUSION BODIES

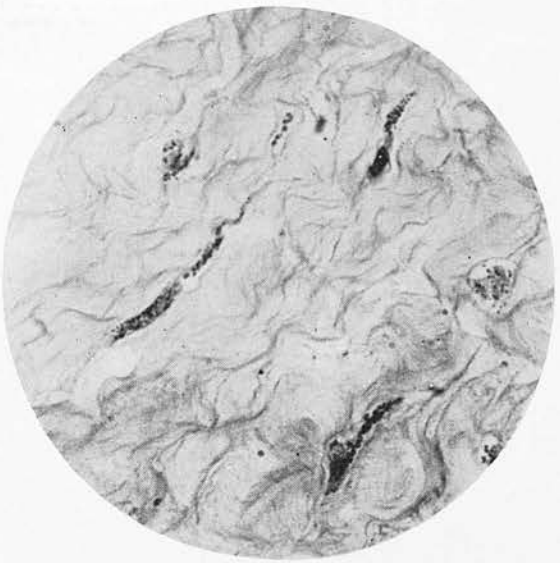


FIG. 1.—Substantia propria of corneal tissue culture infected with vaccinia virus. Two days' growth. Note numerous fibroblasts with intracytoplasmic inclusion bodies. Mann's stain. ×550.

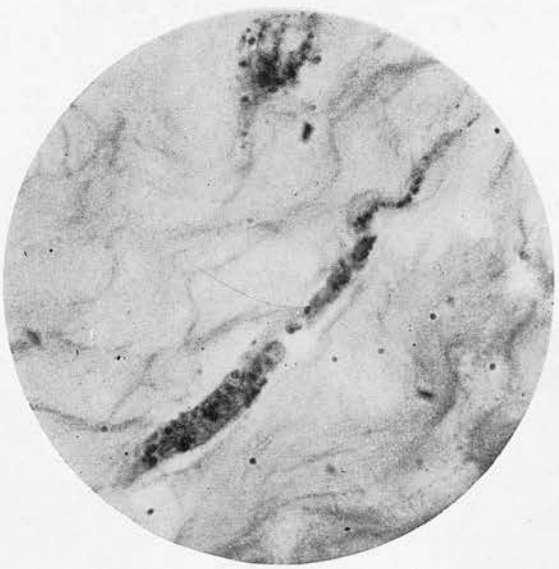


FIG. 2.—Higher power view of fig. 1. Large-sized fibroblast with numerous inclusion bodies. Mann's stain. ×900.

large fibroblasts. At all stages clumps of small granules were found lying free in the spaces of the substantia propria. These resembled in all respects the bodies found intracellularly at the corresponding stage of infection.

Proliferation of the superficial corneal epithelium occurred from the first day and resulted in a marked increase in thickness of this layer, but no Guarnieri nor other inclusion bodies were detected at any time. Rivers, Haagen and Muckenfuss (1928-29, 1929), however, successfully demonstrated Guarnieri bodies in a similar series of experiments.

In the control series fibroblasts in the substantia propria increased in number and in size from the first, but never to the extent noted in infected cultures. Proliferation of the superficial corneal epithelium was also noted. No inclusion bodies nor free granules were observed in the superficial epithelium or substantia propria of these controls.

These results were confirmed in all essential points with a bacteria-free suspension of elementary bodies prepared from ordinary vaccine pulp by Dr D. McClean of the Lister Institute. This was a dermal strain which had not at any time been passed through the testis, whereas our strain had been repeatedly so passed.

Conclusions.

It has proved impossible to infect cultures consisting purely of corneal epithelium, grown by the slide technique, with vaccinia virus in such a way as to retard or otherwise affect the growth of the developing cells. Cultures grown by the tube technique and consisting of all corneal layers were, however, affected. In these cultures inclusion bodies developed in fibroblasts, which increased in number and attained a large size. The inclusions closely resembled those seen in vaccinial keratitis of the rabbit (Rhodes and van Rooyen, 1937).

Tissue culture presents the advantage that the tissue under examination is free from invasion by blood cells ; thus the bodies above described cannot have represented the acidophilic granules of degenerate polymorphs, which may simulate the smaller forms of the inclusions described. As regards the precise nature of these bodies we suggest that they represent aggregates of the actual particles of vaccinia virus, possibly surrounded by a localised acidophilic change in the fibroblastic cytoplasm.

Summary.

1. Tissue cultures from the rabbit cornea consisting purely of epithelial cells showed no departure from normal when exposed to the action of vaccinia virus.

2. In cultures consisting of all corneal layers, fibroblasts growing in the substantia propria developed characteristic inclusion bodies. These cells also increased markedly in number and size.

During the progress of this work A. J. R. has held a Crichton Research Scholarship and an expenses grant from the Moray fund; C. E. v. R. has received an expenses grant from the Medical Research Council. The authors desire to express their thanks to Dr D. McClean for kindly supplying vaccinial elementary bodies.

REFERENCES.

BLAND, J. O. W., AND CANTI, R. G.	1935.	this *Journal*, xl. 231.
FORD, J.	1934.	*J. Roy. Micro. Soc.*, liv. 263.
GOODPASTURE, E. W., WOODRUFF, A. M., AND BUDDINGH, G. J.	1932.	*Amer. J. Path.*, viii. 271.
RHODES, A. J.	1937.	*Edin. Med. J.*, 4th ser., xliv. 410.
RHODES, A. J., AND VAN ROOYEN, C. E.	1937.	this *Journal*, xliv. 357.
RIVERS, T. M., HAAGEN, E., AND MUCKENFUSS, R. S.	1928-29.	*Proc. Soc. Exp. Biol. and Med.*, xxvi. 494.
,, ,, ,,	1929.	*J. Exp. Med.*, l. 665.

PRINTED IN GREAT BRITAIN BY OLIVER AND BOYD LTD., EDINBURGH

CHAPTER IX

TISSUE CULTURE IN THE STUDY OF VIRUSES

CERTAIN tissue cells will readily grow *in vitro* if adequately supported, nourished, and incubated, and such tissue cultures may be used in the study of viruses in various ways. Thus, they have been used in the study of virus inclusion bodies and life cycles, as a specialized culture medium to secure proliferation, and in the study of certain aspects of virus immunity. Before these applications can be considered, however, the methods used to secure growth of normal cells must first be discussed.

Five methods of tissue culture are in general use: (1) the clotted plasma method; (2) Miyagawa's method; (3) the liquid culture method; (4) the tube culture method; (5) Carrel's method. We shall now discuss some general points in tissue culture technique; afterwards, these common methods will be individually described. For fuller details than can be given here, a modern text-book on tissue culture should be consulted (e.g. Cameron, 1935; Parker, 1938).

Source of Cells for Tissue Culture

Epithelium is most readily obtained from the rabbit's cornea as follows: The animal is killed, and the eyelids and surrounding fur cut away; the eyeball is freed in its socket, and held rigidly in volsellum forceps. The cornea is then washed with numerous changes of sterile saline, and lightly cross-hatched with a sterile cataract knife. In this way numerous small squares of corneal epithelium are outlined, which can be detached by slicing with the flat of the knife. These small pieces are placed in a covered bath of Tyrode's solution, and incubated at 37° C. until required.

Fibroblasts can be readily obtained by culturing small pieces of such parenchymatous organs as the lung, liver, kidney, or heart. We have obtained excellent growths of fibroblasts from mouse lungs.

Testis. Minced rabbit testis is frequently employed; mouse testis may also be used.

Chick embryo cells. Epithelium may be obtained from the lung, and fibroblasts from the leg muscles, of the chick embryo. The whole embryo is frequently used in media of the Maitland type.

Mouse and rat embryos may be used. They are removed 2 to 3 days before term and finely minced.

Materials required for Tissue Culture

Slides measure 3 by 1½ in. with a central hollow of 1 in. diameter, and should be wrapped in Kraft paper.

Coverslips should be of thickness number 1 and contained in a small tin or glass jar.

Pasteur pipettes have a very fine point and are contained in a tin.

Watch-glasses are wrapped singly in Kraft paper.

Instruments: a selection of seekers, forceps, and probes are contained in a tin box.

All the above are sterilized in the hot-air oven.

Glass tubes measure 3 in. long by ½ in. in diameter; they are corked at either end by a rubber bung, and should be sterilized by autoclaving.

Incubator: this should be set at 37° C., contain numerous rigid trays, and have a moist atmosphere.

Tyrode's solution is prepared as follows: sodium chloride, 3 gm.; potassium chloride, 0·2 gm.; dextrose, 1 gm.; acid sodium phosphate, 0·05 gm.; sodium carbonate, 1 gm.; magnesium chloride (5 per cent. anhydrous salt), 4 c.c.; calcium chloride (5 per cent. anhydrous salt), 4 c.c.; distilled water, 1,000 c.c.

This strongly alkaline solution is adjusted to pH 7·4 by addition of a 5 per cent. solution of 85 per cent. phosphoric acid, approximately 1 to 1·5 c.c. being required per 1,000 c.c. of solution. Acidity should be corrected by addition of 10 per cent. sodium carbonate solution. The solution is finally sterilized by filtration through a single disk of a Seitz (EK) filter at 30 mm. of mercury; it is then distributed aseptically into small bottles and tubes, and stored in the ice-chest until required.

Sealing mixture consists of vaseline, 7 parts, and paraffin (m.p. 54° C.), 1 part; it is sterilized by placing a small screw-topped bottleful in boiling water.

Nutrient fluids: Plasma. Chicken plasma is recommended, and is obtained by bleeding a young bird which has been starved for 24 hours. The wing is carefully plucked and sterilized, and the vessels in close relation to the humerus are located. Two ligatures about $\frac{1}{3}$ in. apart are then passed loosely round the vessels, which are cut in between these points. As the blood flows out it is received into a series of test-tubes containing heparin to prevent clotting. After sufficient blood has been collected, the ligatures are tightened and bleeding arrested. The bird should receive a large dose of saline solution given subcutaneously to make up for the loss of fluid. Rabbit plasma may also be used, the heparinized blood being centrifuged, and the plasma separated.

It has been found that mammalian plasma can be stored for a long time in the ice-chest, if treated in the following way (Baroni, 1930, and personal communication): Whole blood is mixed with Tyrode's solution (10 to 15 times concentrated), and then centrifuged. The plasma is separated and stored. When serum is required for use, 1 part of plasma is mixed with 3 or 4·25 parts of distilled water, when a clot forms.

Rabbit serum. A rabbit is heart-punctured, and the blood allowed to clot, the serum being separated after 'ringing' and centrifugation.

Spleen extract. A freshly removed rabbit's spleen is ground-up with approximately 10 parts of Tyrode's solution. After low-speed centrifugation for $\frac{1}{4}$ hour the clear supernatant is removed from the deposit, and constitutes spleen extract, which may be stored in the ice-chest if so desired. We recommend this extract, although embryo extract can also be used.

Embryo extract. The egg-shell of an 8- to 10-day chick embryo is carefully disinfected with spirit and iodine, and when dry is partially cut away with sterile scissors. The embryo is removed, then minced and ground-up with 3 c.c. of Tyrode's solution. After slow centrifugation for a few minutes, the supernatant fluid is withdrawn to constitute embryo extract.

METHODS OF TISSUE CULTURE

1. The Clotted Plasma Method

(*a*) Preparation.

For speed and accuracy the following routine is recommended, the layout of the culture table being as shown in Fig. 17:

1. Three working places are desirable, the workers sitting side by side on the far side of the table, facing the reader.

2. The worker on the reader's right is responsible for unwrapping

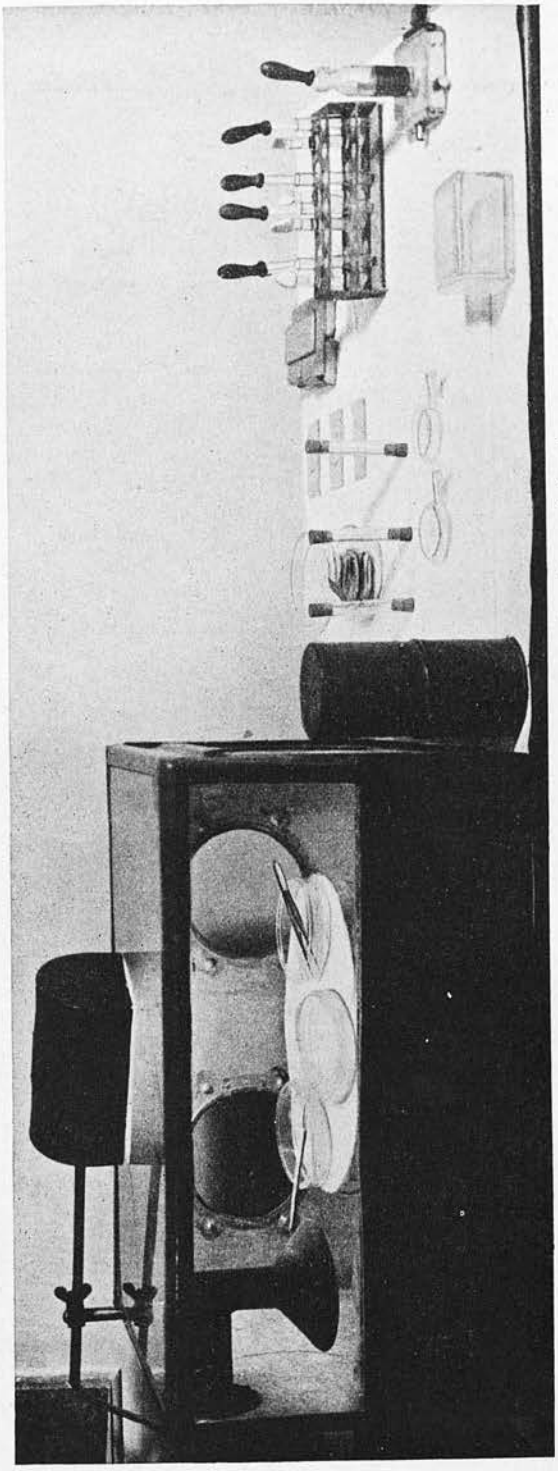


FIG. 17. Showing the layout of apparatus for tissue cultivation.

sterile hollow-ground slides, ringing round the hollow with sealing mixture (kept melted by a water-bath or electric hot-plate), and passing them on to the central worker.

3. This worker mixes up the nutrient fluids in the following proportions: chick plasma, 2 parts, rabbit serum, 2 parts, and spleen extract, 1 part; this is carried out by pipetting the correct number of drops of each material

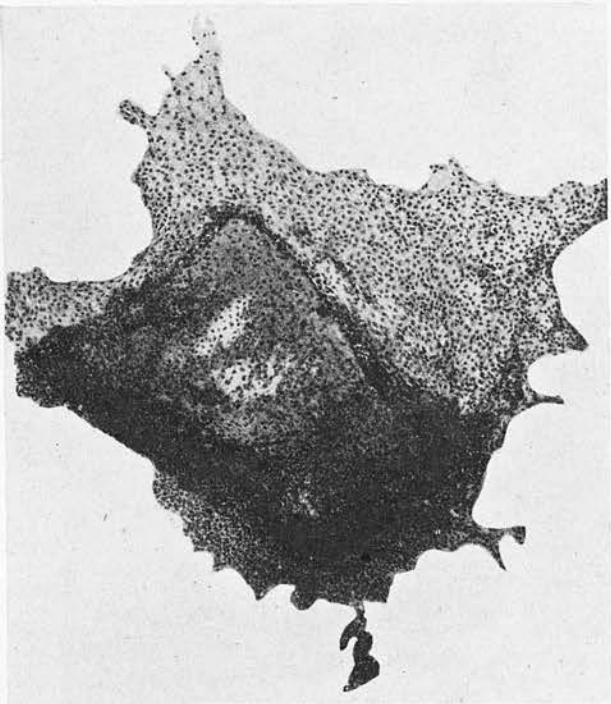


FIG. 18.[1] Two days' growth of epithelium from rabbit cornea. Note darkly staining central area whence new cells have grown out. ×45. Stained Haematein and Eosin.

into one of the sterile watch-glasses. The contents are mixed by sucking up once or twice in another sterile Pasteur pipette.

4. Meanwhile, the worker on the reader's left, working with his hands inside the box, is placing 2 to 3 squares of cornea, or other tissue, onto sterile coverslips. These pieces of tissue are referred to as 'explants'.

5. The central worker, working through the side door of the box, covers each piece of tissue with a drop of nutrient fluid, and then picks up each slip with a vaselined slide. These are left upside down for a few minutes, until the plasma clots, when they are inverted and placed on trays in the incubator.

(*b*) Progress.

Cultures may be examined microscopically after 36 to 48 hours, when, if successful, a considerable growth should be apparent. Epithelium spreads out as a continuous sheet around the explant, and has a very regular growing edge (see Fig. 18). Each cell is roughly polyhedral in shape

[1] Figs. 18–22 are reproduced by permission of the *Edinburgh Medical Journal* (see vol **44**, p. 410).

and rather granular in appearance (see Fig. 19); by appropriate methods much of this granularity can be shown to be due to fat (see Fig. 20). Fibro-

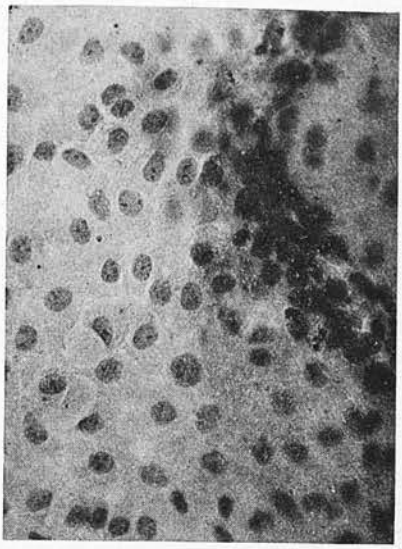


FIG. 19. Higher-power view of Fig. 18. Note typical epithelial cells with spherical nuclei. ×300. Stained Haematein and Eosin.

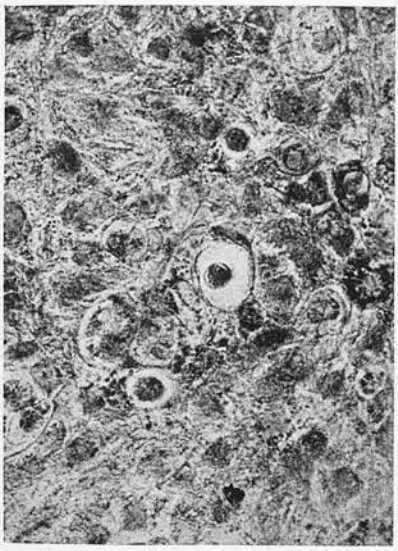


FIG. 20. Growth of corneal epithelial cells. Note darkly staining globules of fat in cytoplasm of numerous cells. ×350. Stained Sudan III and Haematein.

FIG. 21. Mouse lung culture. Note typical fibroblasts with oval nuclei and elongated processes. ×850. Stained Haematein and Eosin.

blasts grow out more in the form of individual strands and filaments, thus giving the edge of the culture an irregular appearance (see Fig. 21). Cells growing horizontally appear long and spindle-shaped, while those growing

vertically appear polyhedral, being seen in cross-section. The culture close to the explant will probably show some leucocytes and mononuclears, but this is of no consequence as pure cultures are not essential in virus work.

Cultures may be infected at the time of setting-up, or later. Bland and Robinow (1939) infected their cultures after about 3 days growth, when the culture medium had partly liquefied. In these experiments, the cultures were washed with saline to remove excess virus inoculum, and then nutrient fluid was added and the growths remounted.

(*c*) Subcultivation.

Growth should occur for 5 to 7 days, before the nutrient fluid is exhausted; the results of most virus experiments should be evident by then, but occasionally it may be necessary to subculture. If so, the slip is removed from the slide, and placed, culture upwards, on a dark background. The clot is then cut through the centre with a sterile razor blade, down to the glass. The old clot is picked off, and the two culture fragments are floated out in a warm bath of Tyrode's solution. Two fresh cultures can then be prepared by covering these pieces with nutrient fluid on a coverslip, as already described.

(*d*) Histological examination.

Cultures embedded in a solid plasma clot are difficult to stain, as fixation frequently results in the formation of an insoluble precipitate. The following method, which dispenses with previous fixation, can be recommended:

1. Haematein and Eosin Method (see Rhodes, 1937).

Solutions required.

Haematein: methylated spirit, 75 c.c.; glacial acetic acid, 5 c.c.; formalin, 10 c.c.; Mayer's acid haemalum, 5 c.c.

Alcoholic eosin: saturated solutions of alcoholic eosin and methylated spirit, equal parts.

Procedure.

1. Remove slip; float, culture down, in a bath of haematein for 10 minutes.
2. Wash in methylated spirit and then in tap water.
3. Dehydrate partially with methylated spirit and absolute alcohol.
4. Counterstain in alcoholic eosin for 1 minute.
5. Wash with absolute alcohol, and leave in xylol till dehydration is complete. Mount in balsam.

This method stains nuclei blue and cytoplasm pink. A number of other methods may also be used; most of these involve preliminary fixation.

2. Preliminary Treatment with Warm Fluid.

It is said that if the slip be floated, culture downwards, in a bath of warm Ringer's or Tyrode's solution for approximately 30 minutes, the plasma becomes detached, and even if not, subsequent fixation and staining will be rendered easier.

3. Fixation Methods.

Fix the culture, after the above treatment, in iodine vapour, formalin or osmic acid. Another method is to fill-up the hollow of the slide with 2 per cent. formalin in saline, and then float the slip, culture downwards, on the surface.

4. Dehydration and Embedding.

Thereafter, the culture may be sliced off the slip with a sharp razor blade, dehydrated, cleared, and embedded in paraffin. Sections are cut parallel to the axis of the slip and may be stained by any suitable method.

5. Fischer's Methods (see 1925).

Method (a). Procedure.

1. Fix in 2 per cent. formalin in Ringer's solution for 1 hour.
2. Wash in running water for 3 hours, and distilled water for 1 hour.
3. Place in 10 per cent. aqueous haematoxylin for 10 to 12 hours; wash for ½ hour.
4. Treat with 50, 75, 95 per cent. alcohols for 5 minutes each.
5. Place for 10 minutes in each of the following mixtures: 95 per cent. acetone and 5 per cent. xylol, 70 per cent. acetone and 30 per cent. xylol, and 30 per cent. acetone and 70 per cent. xylol.
6. Clear in xylol, and mount in balsam.

Method (b). Solution required.

Saturated alcoholic solution of methylene blue, 30 c.c.; 1/10,000 potassium hydroxide, 100 c.c.; ripen in incubator for 1 month.

Procedure.

1. Fix and wash as above.
2. Apply distilled water for 5 minutes.
3. Pour on methylene blue, heat till steam rises, leave for 15 minutes; wash.
4. Dehydrate and clear as above, except that for alcohols 1 minute is enough, and for acetone-xylol mixtures 2 minutes each.

6. Strangeways's Method (see 1924).

Solutions required.

Acetic alcohol: 0·5 per cent. glacial acetic acid in 70 per cent. alcohol.
Iron alum: 2·5 gm. iron alum in 100 c.c. distilled water.

Procedure.

1. After treating in warm fluid, as above, place slip in a watch-glass of acetic alcohol for 5 minutes; wash in running distilled water for 5 minutes.
2. Apply iron alum for 30 minutes; wash as in 1.
3. Float slip, culture down, on the surface of ½ per cent. watery haematoxylin for 5 minutes at 37° C.; wash for 2 to 3 minutes.
4. Differentiate in iron alum, control microscopically; wash as in 1.
5. Mount in Farrant's mountant.

7. Stain for Fat (Strangeways, 1924).

Procedure.

1. Proceed as in above method, but after differentiation in alum and washing, place in saturated solution of either Scharlach R or Sudan III for 5 minutes; wash in water.
2. Mount in Farrant's mountant.

8. Bland and Robinow's (1939) Method.

Procedure.

1. Float cultures (on glass mounts) for a minute on warm saline, and then expose to a strong concentration of osmic acid for 5 to 6 minutes.

2. Harden in 70 per cent. alcohol for 5 minutes; wash in distilled water.
3. Stain overnight in Hollborn's Giemsa (20 drops to 15 c.c. buffered distilled water, pH 6·9).
4. Differentiate in acetone-xylol mixtures; before this, weak acetic acid should be applied to correct over-red preparations.

2. Miyagawa's Method (Miyagawa *et al.*, 1936)

This is a variation of the usual clotted plasma method, and has been used to study the development of the granulo-corpuscles of lympho-granuloma inguinale (L.G.I.).

(*a*) *Preparation.*

Guinea-pig plasma (heparinized) and mouse spleen extract are mixed in the hollow of a hollow-ground slide; this forms a thin film, which soon clots. Fragments of normal mouse testicle or spleen are embedded in the clot, alongside fragments of a mouse brain infected with L.G.I. The whole is then covered with more spleen extract, sealed, and covered with a mica slip.

(*b*) *Progress.*

New cells can be observed to grow out of the normal fragment, which gradually becomes infected with virus from the neighbouring brain tissue.

(*c*) *Subcultivation.*

This is carried out by removing the liquid extract and infected brain tissue, washing the testicle or spleen fragment in Tyrode, and embedding it in another plasma clot. Some healthy testicle or spleen is now implanted beside this infected fragment, and the whole covered and sealed as before.

(*d*) *Histological examination.*

The liquid extract is removed; the tissue fragments are then fixed in formalin, and stuck to an albumenized slide. Giemsa's stain shows typical granulo-corpuscles in the newly formed cells at the edge of the fragment.

3. The Liquid Culture Method

(*a*) *Preparation.*

These cultures are set up in the same way as in the clotted plasma method, except that the nutrient fluid varies. Thus, the cells are grown in mixtures of serum and Tyrode's solution, or plasma and serum, without added extract. Generally speaking, the growth of cells obtained by this method is poor. The method must, however, be used for micromanipulation studies, as the needles cannot work through clotted plasma.

(*b*) *Progress.*

Progress is followed microscopically, great care being taken not to shake or jolt the culture.

(*c*) *Subcultivation.*

The exhausted nutrient fluid is simply sucked off, and replaced with a fresh supply.

(*d*) *Histological examination.*

To those methods already mentioned in previous sections may be added one other:

GIEMSA'S METHOD (Bland and Canti, 1935).

Buffer solution required: potassium dihydrogen phosphate, 1 gm.; disodium hydrogen phosphate, 2 gm.; distilled water, 1,000 c.c.

Stain: 2 drops of Giemsa in 1 c.c. of buffer solution.

Procedure.

1. Gently wash culture in saline, and fix in methyl alcohol for 5 minutes.
2. Rinse in buffer solution, remove, and discard the centre of the explant.
3. Stain for 24 hours at room temperature.
4. Differentiate in acetone, clear in xylol, and mount in balsam.

4. The Tube Culture Method

This method has been used in studies on vaccinial inclusion bodies (Rivers *et al.*, 1928–9, 1929 *b*; Rhodes and van Rooyen, 1937).

(*a*) *Preparation.*

A rabbit's eye is freed in the orbital cavity; the cornea is washed with saline, and dissected out entire. It is then cut up in a bath of Tyrode's solution into 8 to 12 squares of approximately equal size. Nutrient fluid, as used in the clotted plasma method, is then pipetted into each of a number of glass tubes (see Fig. 17) to a depth of $\frac{1}{2}$ in.; these tubes are closed at either end by a rubber bung. When the clot has formed, a piece of cornea is placed horizontally on top, and covered with another layer of fluid. Soon the contents of the tube have formed into a solid clot, in the centre of which lies the piece of cornea.

FIG. 22. Corneal tissue culture by tube technique. Note thickening of superficial corneal epithelium owing to cellular proliferation. ×800. Stained Haematein and Eosin.

(*b*) *Progress.*

This can only be estimated by examining histological preparations.

(*c*) *Subcultivation.*

This cannot be carried out, but the tissue should grow for at least 7 days before the nutrient fluid is exhausted.

(*d*) *Histological examination.*

1. Remove both stoppers, gently tap the tube, and the entire clot should slip out. Fix in Helly's or other fixative for a short time.

2. Thereafter, dehydration, clearing, and embedding are carried out as usual. Paraffin sections are cut parallel to the sides of the tube, thus giving, in the centre of the clot, a radial section of cornea.

3. Sections may be stained by any desired method, e.g. Mann's stain.

This method has an advantage over the slide methods, for a perfect histological preparation is obtainable. Such sections show that cellular proliferation has occurred in the depths of the cornea, as well as in the superficial epithelium. Thus, the fibroblasts in the substantia propria increase in numbers, and there is considerable thickening of the epithelial layers (see Fig. 22).

5. Carrel's (1923) Method

(*a*) *Preparation.*

Cultures are contained in special Carrel flasks, of which various shapes have been designed. The commonest shape for bacteriological purposes is Type D (see Fig. 17). The diameter of these flasks may be either 5 or 8 cm. The other shapes are: Type A, resembling D in shape, but fitted with a top opening, 3 cm. in diameter; Type B, resembling D, but fitted with two side arms; Type C has a longer neck than D, and top and bottom openings; Type E resembles D, but has a bottom opening covered with mica.

The flask is inoculated by introducing 0·5 c.c. of plasma, which is run uniformly over the bottom; 1·5 c.c. of Tyrode's solution (with 5 per cent. tissue extract added) is then mixed with the plasma. The fragments of tissue to be cultured are embedded in the plasma just before it clots, by using a long narrow metal spatula. When the clot forms, some fluid medium is run over the surface; if the cells are epithelial or fibroblastic, use 25 per cent. tissue extract in Tyrode, and if leucocytic, use 2 parts Tyrode and 1 part each of serum and tissue extract (Cameron, 1935).

(*b*) *Progress and* (*c*) *Subcultivation.*

The fluid requires to be changed every 2 to 5 days; to do this, carefully flame the neck of the flask, suck out the old fluid, and add fresh. When the original clot becomes exhausted a section is cut out with a spatula, the tissue dissected out and re-embedded in a new flask.

(*d*) *Histological examination.*

Sections of clot may be removed with a spatula, fixed and taken through to paraffin, excellent results being obtained after suitable staining. In other cases, fixative may be poured into the flask, and the fixed tissue removed by breaking the glass.

Having discussed the various methods whereby normal cells may be grown *in vitro*, it remains to describe their applications to the study of viruses.

THE STUDY OF VIRUS INCLUSION BODIES AND LIFE CYCLES

Various viruses produce their characteristic inclusions when added to, or incorporated in, tissue cultures. Thus, with slide methods, lymphogranuloma inguinale (see p. 186) and vaccinia (see p. 323), also psittacosis (see p. 588), have been shown to form inclusions. The virus of herpes has been studied by the tube culture method and inclusion bodies described (Rivers *et al.*, 1928–9, 1929 *b*). Rhodes and van Rooyen (1937), employing the same method in the study of vaccinia, found that inclusions developed. Carrel's method has been used by Andrewes to demonstrate inclusion bodies in virus III of rabbits (1929 *a*, *b*), and herpes (1930).

With regard to tissue culture in the study of life cycles, the following method was used by Bland and Canti (1935), working with psittacosis virus:

1. The nutrient fluid was prepared as follows: 2 parts of chick plasma were mixed with 3 parts of chick embryo extract (2·5 c.c. saline to each minced 10 days' embryo); when clotting occurred, fluid was expressed. This fluid was diluted with an equal volume of saline, and centrifuged at high speed for 1 hour, to remove refractile granules. Epithelial cells were cultured directly in this fluid, but fibroblasts were first grown by the clotted plasma method, and subcultured into this later.

2. Explants of the muscle or lung tissue to be cultured were mounted by Maximow's flying coverslip technique, a no. 1 round slip ($\frac{7}{8}$ in. dia-

meter) being fixed to a larger square slip ($1\frac{1}{4}$ in.). The cells were then covered with a small amount of nutrient fluid, and the slips inverted over a hollow-ground slide.

3. Cultures were infected by adding virus to the nutrient fluid at the onset, or after a certain amount of growth had occurred. In the latter case, the medium was replaced by a drop of virus, and the culture incubated for 1 hour. Then the culture was re-opened, the virus washed off, and fresh nutrient fluid added.

4. Such infected preparations were examined after a further period of growth, either by Giemsa's stain, or by dark-ground microscopy. For the latter examination cultures were mounted as contact specimens. The thick centre of the culture was discarded and the slips were then inverted over a thin slide to which thin strips of coverslip were luted with distilled water.

In this study the various developmental phases in the life-history of the psittacosis virus were worked out. Somewhat similar studies have recently been carried out with vaccinia (Bland and Robinow, 1939).

THE PROPAGATION OF VIRUSES

Ever since viruses were first discovered, at the close of last century, attempts have been made to culture them. Although viruses resemble bacteria in many respects, they differ radically in that they cannot be cultivated on ordinary laboratory media, however much enriched. The whole trend of modern work shows that viruses will only grow in the presence of living cells, and it has been suggested that they exhibit the most extreme degree of parasitism in nature.

Prior to 1928, a number of investigators had obtained growths of vaccinia virus in tissue culture; the usual method was to use hanging-drop preparations of embryo tissue or testis suspended in serum or plasma, with virus added. This technique was somewhat uncertain; it did not permit of large-scale operations, nor of ease and accuracy of titration. A real advance was initiated when Maitland and Maitland (1928) grew vaccinia virus in a medium containing hen's kidney and serum.

Maitland's original medium.

The inoculum consisted of the testis of a rabbit inoculated 4 days before with commercial vaccine lymph, ground in sand, and M/50 phosphate (pH 7·6) added. After centrifuging, the supernatant fluid (approximately 5 c.c.) was used for the primary inoculum.

The kidneys of a hen were cut up with scissors and 0·66 c.c. placed in a flask, 1·33 c.c. of virus inoculum (diluted 1/6·6 with Tyrode's solution) being added. After standing in the cold room for 4 hours, 12 c.c. Tyrode and 6 c.c. hen serum were added, thus making a final dilution of virus inoculum of 1/100. Thereafter, 2 c.c. amounts were distributed into Carrel flasks (Type D), and incubated aerobically at 37° C., without caps. Cultures were titrated for the content of virus by grinding with sand, centrifuging, and injecting 0·2 c.c. of dilutions of the supernatant intradermally in rabbits. During four successive subcultures virus increased approximately 25×10^6 times. In these cultures it was not possible to detect any tissue growth; after 24 hours the smallest pieces of kidney had begun to disintegrate, this process of autolysis being complete by the third day.

Maitland's later media.

Maitland and Laing (1930) substituted rabbit for hen tissues; they placed 0·33 c.c. of freshly minced kidney or testis into a wide tube.

Vaccinial material was then added and gently mixed; 3 c.c. of serum, and enough Tyrode's solution to give a final bulk of 10 c.c., were then added (serum last). The mixture was finally distributed into Carrel flasks, in 2 c.c. amounts. These workers found that vaccinia would not multiply in liver or spleen cultures.

Maitland, Laing, and Lyth (1932) found that vaccinia would grow readily in a medium consisting of minced chick embryo and Tyrode's solution, without serum.

Other media of similar type.

1. *Findlay's (1928) method.* In this medium fowl-pox virus was added to the skin and brain of a 12-to 15-day chick embryo; after standing in the ice-chest for 2 days, equal amounts of embryo-virus suspension were distributed into flasks containing 5 c.c. chick plasma, and incubated at 37° C. for 4 days.

2. *Smith's (1935) method.* Minced 8- to 10-day chick embryos were introduced into Carrel flasks in amounts from 0·05 c.c. to 0·1 c.c. The virus (influenza) inoculum was dropped over the tissue; it was left for 10 minutes, and then 2 c.c. of Tyrode's solution was added. The flask was rotated to distribute its contents, and incubated at 37° C. Subcultivation was carried out by transferring 0·05 c.c. of fluid from flask to flask.

3. *Tamura's methods* (1934, 1935; D'Aunoy *et al.*, 1935). A small quantity of virus-containing material was added to 10 c.c. of Tyrode's solution, in which was either a piece of guinea-pig kidney or liver, or rabbit kidney.

4. *Dochez, Mills, and Kneeland's (1936) method.* Minced 10-day chick embryo was pipetted into two culture tubes (2 cm. diameter) in equal amounts; 10 c.c. of special peptone broth was then added (sodium chloride free, made with non-toxic casein peptone, and containing 0·1 per cent. gelatin). Sufficient cysteine hydrochloride was then added to give a concentration of 1/2,000 (cysteine was prepared in 1 per cent. solution, neutralized with caustic soda, autoclaved, and kept under a vaseline seal). The culture tubes were sealed with vaseline, and stored at 4° C. till required. Cultures were initiated by introducing virus-containing material (common cold) through the vaseline seal. Subcultures were carried out by transferring 1 c.c. of fluid to a fresh culture tube, at intervals of from 2 to 9 days.

5. *Li and Rivers's (1930) method* (see also Rivers and Ward, 1933 *c*). This method was introduced to grow vaccinia virus on a large scale. The medium consisted of rabbit testis, rabbit serum, and Tyrode's solution for primary growths; after subculturing, chick embryo and Tyrode's solution were substituted. The conical flask made to contain this medium was of an unusual shape, being fitted with a side ventilating arm and a gutter neck to catch condensation water. The flasks were made in two sizes to hold 17 c.c. and 6 c.c. respectively.

6. *Webster and Clow's (1936, 1937) method.* To grow rabies virus in bulk, these authors use a mixture of Tyrode's solution (9 parts) and monkey or horse serum (1 part); 4 c.c. is placed in a 50 c.c. Erlenmeyer flask, and mouse, rabbit, or chick brain added. Rat or mouse embryo, with chick plasma, may be successfully used also (Bernkopf and Kligler, 1937).

Viruses grown in tissue culture.

The following viruses of human diseases have all been grown in tissue cultures of the types just described, and definite propagation reported: common cold (p. 525); herpes febrilis (p. 161); influenza (p. 554); Japanese

encephalitis (p. 868); lymphogranuloma inguinale (p. 187); poliomyelitis (p. 798); psittacosis (p. 588); rabies (p. 678); Rift Valley fever (p. 416); St. Louis encephalitis (p. 860); vaccinia (p. 328); yellow fever (p. 485); varicella (p. 256).

Various animal viruses may also be grown in tissue culture: e.g. ectromelia; foot-and-mouth disease; louping-ill; pseudo-rabies; and virus III of rabbits.

Attempts to grow vaccinia in cell-free medium.

The comparative ease with which vaccinia virus grew in Maitland's and similar media led Eagles and his associates to attempt its cultivation in cell-free media. Various series of these experiments were carried out:

1. Eagles and McClean (1930, 1931) prepared a kidney extract as follows: a rabbit kidney was removed aseptically and very finely minced in 3 to 4 c.c. of Tyrode's solution. After high-speed centrifugation for 20 minutes the supernatant fluid constituted kidney extract; this was examined for cells, but none could be demonstrated. The medium, designated 'cell-free', was prepared from this kidney extract, vaccinia virus, and Tyrode's solution, with or without the addition of rabbit's serum; incubation was carried out in Carrel flasks. Vaccinia virus increased considerably in amount in these cultures; e.g. in one case thirteen subcultures showed an approximate virus increase of 10^{43}, although the approximate dilution of the original seeding of virus was 10^{-26}.

2. Eagles and Kordi (1932) prepared another type of extract. A rabbit kidney was chopped and ground-up in 2 c.c. of 9 per cent. (hypertonic) saline, the suspension being divided into two equal portions in centrifuge tubes. One tube was left at room temperature for at least 1 hour, while the other was frozen at $-12°$ C. to $-13°$ C. for 1 hour. The frozen tube was then thawed in water (80° C.). By this means it was hoped to carry further the cellular disintegration begun by hypertonic saline. Sterile distilled water was then added to each tube, to make the concentration of saline normal, and centrifugation at 4,000 r.p.m. carried out for 1 hour. The supernatant fluid was likewise centrifuged; when stained films were examined no whole cells were seen, only a few possible fragments.

Equal parts of fresh rabbit serum and Tyrode's solution were added to the kidney extracts to make a dilution of 1/50 of virus seeding, two parallel experiments being set up. In the first, the kidney extract treated only with hypertonic saline was used; in the second, the frozen and thawed extract was employed. The virus preparation was a fresh testicular strain of neurovaccine; it was diluted 1/50 in the medium, which was then thoroughly mixed, and distributed in 2 c.c. amounts in Carrel and Rivers flasks. Incubation was carried out at 37° C. for 3 to 6 days. In one unfrozen culture, in the course of subcultivation, a total virus multiplication of 10^{20} was obtained, although the dilution of the original virus seeding was 10^{-17}. Virus increase in the frozen medium was most irregular, and it was concluded that a substance released only from fresh cells was essential for the growth of vaccinia virus.

3. Eagles (1935) has repeated some of his previous work. As kidney extract he used the unfrozen extract of Eagles and Kordi (1932). He concluded that there was poor survival of the virus in large numbers of individual flasks, and an inability to secure either growth or survival of the virus in a number of subcultures. Multiplication did, however, take place in two of the series of subcultures.

This work has never been confirmed, and it is the opinion of numerous workers that vaccinia will only grow in a medium containing some living

cells (e.g. Craciun and Oppenheimer, 1926; Maitland, Laing, and Lyth, 1932; Rivers and Ward, 1933 *a*, *b*; Haagen, 1933). It should be noted that Rivers and Ward (1933 *b*), after treating rabbit testis and chick embryo as described by Eagles and Kordi (1932), found some cells not only alive but capable of growth.

The mechanism of virus-growth in tissue culture.

As regards the manner and site of virus-growth in tissue culture, there has been considerable discussion. Thus, Maitland and Maitland (1928) thought that there were no living cells in their original medium, growth occurring in the presence of dead or dying tissue. It was, however, soon found that living cells were in fact present (Rivers, Haagen, and Muckenfuss, 1929 *a*; Maitland, Laing, and Lyth, 1932). Prior to the introduction of Maitland's medium, it had been shown by Parker and Nye (1925) that growth of vaccinia took place in intimate connexion with the cellular, rather than the fluid, elements of their cultures. These workers prepared tissue cultures of vaccinia virus by the slide technique, rabbit testis being embedded in the centre of a large plasma clot. Tests showed the presence of virus in the testis with its corresponding absence from the plasma. Later, Muckenfuss and Rivers (1930) suggested that at least one function of the living cells of a tissue culture might be to elaborate a diffusible substance which supported the life of viruses, for they observed that vaccinia remained active in a serum-Tyrode mixture separated by a collodion membrane from a serum-Tyrode suspension of living kidney cells. Vaccinia virus incubated in a serum-Tyrode mixture without living cells lost its activity more quickly than the preparation in which living cells were present.

Perhaps the factor postulated by Muckenfuss and Rivers serves to keep virus alive, while more intimate cellular contact, as suggested by the work of Parker and Nye, is needed for actual proliferation. The work of Eagles and his associates, who claim to have grown vaccinia in cell-free media, if confirmed, would suggest that a substance similar to that of Muckenfuss and Rivers can be liberated from tissue, and can support actual proliferation of virus even when the cells producing the factor have been destroyed. No such confirmation has yet been reported, however, and it appears that the growth of viruses in tissue culture is intimately dependent on the presence of living cells.

OTHER APPLICATIONS OF TISSUE CULTURE

In addition to their use in the study of virus morphology and for securing virus propagation, tissue cultures can be used for certain other purposes:

1. *Immunization.* Tissue cultures may be used to furnish a growth of virus suitable for immunization of animals, and of man. Thus cultures of influenza, common cold, yellow fever, and vaccinia viruses have all been used for human immunization (see pp. 516, 525, 489, 374). Rivers and Ward (1935), for example, give the following instructions for preparation of vaccinia virus suitable for use in human vaccination:

(*a*) Routine cultures are ground up in a mortar and inoculations carried out for sterility.

(*b*) Bacteria-free suspensions are mixed with equal parts of sterile glycerol, and stored at temperatures below 0° C.

(*c*) For use, 0·1 c.c. of glycerolated culture virus, diluted 5 to 10 times with sterile saline, is injected intradermally in the skin of the upper arm or thigh, with a 27-gauge needle.

Such cultures have several advantages over the more commonly used vaccines prepared from animal tissues. Thus, they can be guaranteed to be sterile; there is a minimum of foreign animal tissue present; and finally, in an emergency, a large supply can be rapidly obtained by inoculating a sufficient number of cultures from a stock continually passaged in the laboratory.

2. *Isolation of virus.* It has been reported that influenza virus may be isolated directly from patients by inoculation of tissue cultures with nasopharyngeal secretion (see p. 554).

3. *For* in vitro *neutralization tests.* It has recently been shown that the virus-neutralizing power of immune sera can be titrated by making use of tissue cultures (Magill and Francis, 1938). These workers carried out studies with the virus of influenza, which will now be described: into 50 c.c. Erlenmeyer flasks were pipetted 4 c.c. of Locke's solution and 5 drops of minced 13-day chick embryos. Rabbit antiserum (0·5 c.c.) was then added. Finally, 0·5 c.c. of cultured virus was added. Incubation was carried out at 37° C. for 48 hours. After this time, 0·5 c.c. of supernatant fluid was inoculated into a fresh flask containing saline (4·5 c.c.) and minced embryo (5 drops) only. Now, the final dilution of the original serum was at least 1/100. Incubation was carried out as before, and presence of virus tested by intranasal inoculation of lightly anaesthetized mice. If virus was destroyed in the first series, then naturally no proliferation occurred in the second, the mice being unaffected. Actually they carried out rather more elaborate tests with five strains of virus and appropriate sera, and were able to detect antigenic differences between the strains.

REFERENCES

ANDREWES, C. H. (1929*a*), *Brit. J. exp. Path.*, **10**, 188.
—— (1929*b*), *Brit. J. exp. Path.*, **10**, 273.
—— (1930), *J. Path. Bact.*, **33**, 301.
BARONI, V. (1930), *C.R. Soc. Biol., Paris*, **104**, 599.
BERNKOPF, H., and KLIGLER, I. J. (1937), *Brit. J. exp. Path.*, **18**, 481.
BLAND, J. O. W., and CANTI, R. G. (1935), *J. Path. Bact.*, **40**, 231.
BLAND, J. O. W., and ROBINOW, C. F. (1939), *J. Path, Bact.*, **48**, 381,
CAMERON, G. (1935), *Essentials of Tissue Culture Technique*, Farrar & Rinehart, New York.
CARREL, A. (1923), *J. exp. Med.*, **38**, 407.
CRACIUN, E. C., and OPPENHEIMER, E. H. (1926), *J. exp. Med.*, **43**, 815.
D'AUNOY, R., VON HAAM, E., and LICHTENSTEIN, L. (1935), *Amer. J. Path.*, **11**, 737.
DOCHEZ, A. R., MILLS, K. C., and KNEELAND, Y. (1936), *J. exp. Med.*, **63**, 559.
EAGLES, G. H. (1935), *Brit. J. exp. Path.*, **16**, 188.
EAGLES, G. H., and KORDI, A. H. H. (1932), *Proc. roy. Soc.*, B, **111**, 329.
EAGLES, G. H., and MCCLEAN, D. (1930), *Brit. J. exp. Path.*, **11**, 337.
—— —— (1931), *Brit. J. exp. Path.*, **12**, 97.
FINDLAY, G. M. (1928), *Brit. J. exp. Path.*, **9**, 90.
FISCHER, A. (1925), *Tissue Culture*, Levin & Munksgaard, Copenhagen.
HAAGEN, E. (1933), *Zbl. Bakt.*, Abt. I, orig., **129**, 237.
LI, C. P., and RIVERS, T. M. (1930), *J. exp. Med.*, **52**, 465.
MAGILL, T. P., and FRANCIS, T. (1938), *Brit. J. exp. Path.*, **19**, 273.
MAITLAND, H. B., and LAING, A. W. (1930), *Brit. J. exp. Path.*, **11**, 119.
MAITLAND, H. B., LAING, A. W., and LYTH, R. (1932), *Brit. J. exp. Path.*, **13**, 90.
MAITLAND, H. B., and MAITLAND, M. C. (1928), *Lancet*, **2**, 596.
MIYAGAWA, Y., MITAMURA, T., YAOI, H., ISHII, N., OKANISHI, J., GOTO, T., and SHIMIZU, S. (1936), *Jap. J. exp. Med.*, **14**, 207.
MUCKENFUSS, R. S., and RIVERS, T. M. (1930), *J. exp. Med.*, **51**, 149.
PARKER, F., and NYE, R. N. (1925), *Amer. J. Path.*, **1**, 325.
PARKER, R. C. (1938), *Methods of Tissue Culture*, Hamish Hamilton, London.
RHODES, A. J. (1937), *Edinb. med. J.*, N.S. (4th), **44**, 410.
RHODES, A. J., and VAN ROOYEN, C. E. (1937), *J. Path. Bact.*, **45**, 253.

RIVERS, T. M., HAAGEN, E., and MUCKENFUSS, R. S. (1928–9), *Proc. Soc. exp. Biol., N.Y.*, **26**, 494.
—— —— —— (1929*a*), *J. exp. Med.*, **50**, 181.
—— —— —— (1929*b*), *J. exp. Med.*, **50**, 665.
RIVERS, T. M., and WARD, S. M. (1933*a*), *J. exp. Med.*, **57**, 51.
—— —— (1933*b*), *J. exp. Med.*, **57**, 741.
—— —— (1933*c*), *J. exp. Med.*, **58**, 635.
—— —— (1935), *J. exp. Med.*, **62**, 549.
SMITH, W. (1935), *Brit. J. exp. Path.*, **16**, 508.
STRANGEWAYS, T. S. P. (1924), *Technique of Tissue Culture* in vitro, Heffer, Cambridge.
TAMURA, J. T. (1934), *J. Amer. med. Ass.*, **103**, 408.
—— (1935), *J. Lab. clin. Med.*, **20**, 393.
WEBSTER, L. T., and CLOW, A. T. (1936), *Science*, **84**, 487.
—— —— (1937), *J. exp. Med.*, **66**, 125.

CHAPTER X

THE FERTILE EGG IN THE STUDY OF VIRUS DISEASES

THE fertile egg had been studied in connexion with various problems (see Goodpasture, 1938), before Rous and Murphy in 1911 made use of the developing chick embryo in their study of the Rous sarcoma. Twenty years elapsed, however, before the method was generally introduced into bacteriology by Woodruff and Goodpasture (1931), in their study of fowl-pox virus. In the past few years the technique has been widely adopted, and found to be applicable to the study of a variety of virus problems. In the descriptions to follow we always refer, unless expressly stated to the contrary, to hens' eggs; recently, however, Himmelweit (1938) has recommended ducks' eggs.

GENERAL DETAILS OF EGG TECHNIQUE [1]

Source and supply of eggs.

An arrangement should be made with a reliable poultry farm to supply fertile eggs at regular intervals. As soon as received, these should be washed in soapy water, and placed in an egg incubator at 38° to 40° C. for 8 to 12 days.

Inoculation of eggs.

1. *Goodpasture's method.* This method has been used by Goodpasture and his collaborators (see, e.g., Woodruff and Goodpasture, 1931; Goodpasture, Woodruff, and Buddingh, 1931, 1932; Goodpasture *et al.*, 1935; and see Goodpasture, 1938). Twelve-day eggs are candled, and the site of the chorio-allantoic membrane outlined (*vide infra*, method 4). Paraffin is thinly painted on the shell overlying the membrane. The eggs, resting on plasticine, are then placed in water at 40° C.; this reaches up to, but does not touch, the paraffin. A window of 1–1½ sq. cm. is then cut in the paraffined surface, using a hard steel trocar (ground with a triangular end and sharp point). The shell is then gently levered off and paraffin painted over the more or less undamaged shell membrane beneath; this is then torn and folded over the cut shell, to expose the chorio-allantois. The inoculum is then placed on the chorio-allantois, and a rim of vaseline-paraffin mixture built round the edge of the window, a coverslip being pressed firmly down on the top.

Examination of the chorio-allantoic membrane can be carried out through the window with the plate-culture microscope, from day to day.

2. *Burnet's method.* This modification of Goodpasture's method was originally introduced by Burnet (1933); it has been improved from time to time and the latest method embodies a number of new procedures (Burnet, 1936 *b*). The air-space is first pencilled-in; then an equilateral triangle, with 12 mm. sides, is marked over the area of densest opacity of the embryo. The sides of this triangle are cut through with a rotating disk driven by a dental engine. Two intersecting cuts are made over the air-sac, which is then pierced with a short, four-sided, sharp steel rod. The triangle of shell already cut is then removed and the shell mem-

[1] Those desiring fuller information are specially recommended to read the recent monograph of Stevenson and Butler (1939).

brane slit in the direction of its fibres. Slight suction is applied with a teat to the hole in the air-sac. In this way the egg contents are displaced and an artificial air-space formed between the shell membrane and the chorio-allantois. Inoculation of 0·05 c.c. of material is then made with a sterile Pasteur pipette. The orifice is rimmed and sealed as in Goodpasture's method, while the air-sac hole is closed with paraffin. The egg must be most carefully handled in all subsequent manipulations. A somewhat similar method to this has been used by Bengtson and Dyer (1935). These workers cut only two sides of the triangle, bending back the flap to break the third side. Injections are made through the vaseline after the orifice is covered.

3. *Stevenson and Butler's (1939) method.* The air chamber and a small circle 1 cm. in diameter on the side of the egg (within the opaque area) are pencilled-in. The egg is then placed on plasticine with the inoculation site laterally; the shell over this area is swabbed with rectified spirit and flamed. If the air-sac is to be pierced, the shell over this area also should be sterilized. Using a sterile scalpel a hole is then drilled through the shell of the inoculation site. When the hole is made one should look for the pink chorio-allantoic vessels or slight bleeding; if neither is noted, discard the egg.

The actual inoculation is now carried out by one of the three following methods: (*a*) The inoculum is introduced through the hole with a capillary pipette, very little, if any, pressure being applied to the teat. (*b*) First blow in a puff of air, then the inoculum (using separate pipettes). (*c*) After the lateral opening has been made, the air-sac is punctured; air is then blown into the lateral hole and finally the inoculum. All holes are sealed with paraffin.

4. From personal experience we can recommend the following simple method of inoculating eggs: The egg is candled to see if germination has occurred; this is indicated by a dense shadow at one part of the egg, with a clear semi-transparent zone immediately adjacent. The egg is then rotated before the light until this clear zone occupies the top half of the shell, a pencil mark being made at the uppermost part. The position of the air-sac at one end of the egg is also pencilled. The egg is now thoroughly washed and scrubbed in lukewarm soapy water (40° C.) containing some 2 per cent. phenol. Immediately prior to inoculation, the surface of the egg is still further sterilized by setting alight a few drops of alcohol on the upper surface and painting with 4 per cent. tincture of iodine. The egg should rest on a plasticine ring during the actual inoculation.

The method of choice for inoculation depends on the bulk of the fluid to be injected. If this does not exceed 0·05 c.c., the shell is carefully punctured with the point of a pair of sterile scissors and the material introduced with a syringe and needle to a vertical depth of not more than 3 mm., so that the needle point rests on, but does not penetrate, the chorio-allantoic membrane. Should, however, it be desired to inoculate a larger bulk it is advisable first to puncture the shell over the air-sac to make available more space inside the shell. Sometimes a little haemorrhage may accompany the puncture, but the mortality resulting from this is negligible. Apertures in the shell are sealed with ovals of tissue paper soaked in melted paraffin, and finally painted over with more paraffin. Thereafter, the inoculated egg is incubated at 37° to 39° C. in an incubator containing a trough of water to secure a moist atmosphere.

Opening of eggs.

After 3 to 4 days the eggs are ready for examination. In Goodpasture's and in Burnet's methods the vaseline and cover-slip are removed, and the

egg-shell broken away with sterile forceps until enough access has been obtained. In Stevenson and Butler's method the procedure is as follows: The egg is rubbed over with rectified spirit from the narrow end to the middle. The narrow end is then dipped into rectified spirit down to one third of the egg. The egg is then placed in a metal holder and flamed. The sterile (narrow) end projects beyond the holder, and is cracked with a heavy instrument. The shell is picked off the shell membrane with forceps. The exposed portion of the shell membrane is then peeled off with another pair of forceps, and the chorio-allantois exposed; with a third pair of forceps this structure is torn across. The egg is tilted and the contents delivered; the chorio-allantois remains in the shell and is removed into a sterile petri dish. In the method that we use, the paraffin seal is scraped off; the surface is covered with iodine; the egg is then opened by removing a large oval with sterile scissors. The membrane is closely inspected for macroscopic lesions, and if desired is dissected out. The embryo can then be pulled out of the shell. Aerobic and anaerobic cultures should be made from every egg opened, if the contents are to be studied further.

Examination of the infected chorio-allantois.

The membrane is removed from the shell and pinned out on a board, against a dark background. After fixing in formalin or Zenker's solution, the pocks or other lesions may be counted and examined with a lens or plate-culture microscope. If it is desired to examine paraffin sections of the infected membrane, dehydration and embedding can thereafter be carried out, the membrane being rolled-up into a compact mass.

Another histological method is that of Goodpasture, Woodruff, and Buddingh (1931, 1932):

1. Fix in Zenker's solution for 24 hours.
2. Stain the membrane in 2 per cent. aqueous acid fuchsin for 10 to 30 minutes; wash.
3. Counterstain with Löffler's methylene blue for 30 seconds.
4. Differentiate in absolute alcohol, clear, and mount in cedar oil.

The pathological effects produced on the egg by growing viruses.

Before describing the various effects that may result from the action of viruses on the chorio-allantois, certain control observations must be mentioned. Injections of non-irritating fluids should not cause any more than a slight opacity of the chorio-allantoic membrane, but Burnet (1936 *b*) has described the occasional presence of a traumatic lesion, which is more apt to be found in younger than older eggs. It appears as an irregularly thickened opaque area up to 1 cm. in length with, microscopically, replacement of ectoderm by granulation tissue, and epithelial down-growth marginally.

The histological structure of the normal chorio-allantois should be familiar to those searching for inclusions in infected membranes (see, e.g., Burnet, 1936 *b*). Goldsworthy and Moppett (1935) found eosinophilic intracytoplasmic and intranuclear bodies in the chorio-allantois unaffected by any virus. D'Aunoy and Evans (1937) have also drawn attention to the fact that many changes reported in the literature as being specific for viruses can, in fact, be seen in normal eggs. Thus they found the following changes to be perfectly normal: mesodermal oedema and cellular proliferation; endodermal proliferation with vacuolation of cells, which often contained large eosinophilic bodies, probably red cells or degenerated debris; eosinophilic intranuclear bodies were also found.

The lesions produced in the egg by genuine viruses, however, are very

characteristic, and should not be confused with the appearances just mentioned. Virus dropped on to the chorio-allantois must, unless it be rapidly destroyed, first penetrate the ectodermal layer; here the infection spreads outwards with resultant proliferation of infected cells; thereafter, the lesions may assume three main types (Burnet, 1936 *b*).

1. Proliferation of ectodermal cells outstrips the spreading infection, so that a thickened and cornified ectodermal zone surrounds an infected and necrotic centre. Burnet states that this type of change occurs with the

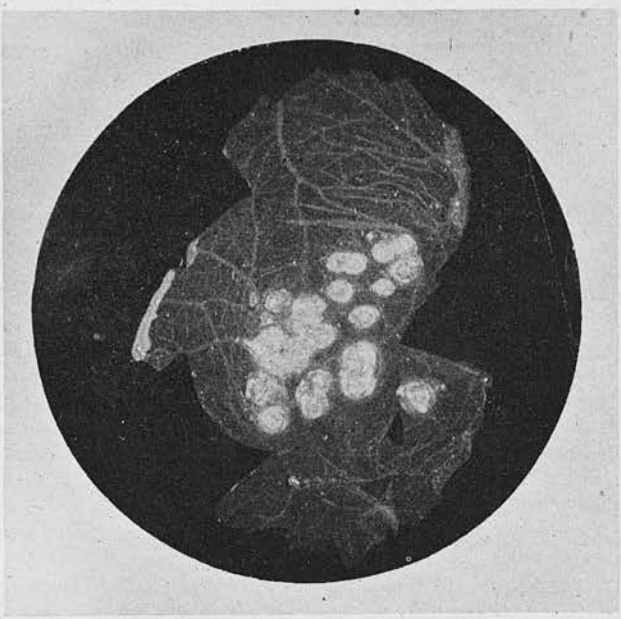


FIG. 23. Shows the appearance of the chorio-allantoic membrane 5 days after inoculation with vaccinia virus; observe the plaques. (By courtesy of Lt.-Col. W. D. H. Stevenson, C.I.E.)

viruses of ectromelia (incubated at 38° to 39° C.), herpes, influenza, louping-ill, and psittacosis. To the naked eye this change is evident as white areas of dense opacity.

2. In this type the lesion is more destructive; much necrosis occurs, with the production of a crater-shaped pock, sometimes with central haemorrhage. This type of change occurs with ectromelia (incubated at 36° to 37° C.), infectious laryngo-tracheitis, and vaccinia (see Fig. 23).

3. The ectoderm does not undergo early necrosis, but proliferation spreads out peripherally, and is followed by infection. A flat conical lesion of semi-transparent appearance, and without central necrosis, results; fowl-pox produces lesions of this type. These lesions are usually described as 'pocks'.

In the milder infections the embryo may not be killed, but in the severer types it is rapidly destroyed.

APPLICATIONS OF THE EGG TECHNIQUE

1. The study of virus morphology.

Numerous workers have found the chorio-allantois a suitable site in which to study the development of inclusion bodies. However, in view of

the above-mentioned findings of inclusion-like bodies in normal membranes, it would appear as if some, at least, of the bodies described were non-specific in character. Inclusion bodies characteristic of the following human diseases have been found in stained preparations of the chorio-allantois: herpes (p. 160); lymphogranuloma inguinale (p. 186); psittacosis (p. 590); vaccinia (p. 322). A number of animal viruses also develop inclusions in this site: ectromelia; equine encephalomyelitis; fowl-pox; Kikuth's canary virus; infectious laryngo-tracheitis of fowls; Newcastle disease of fowls; Pacheco's parrot disease.

Himmelweit (1938) has used an ingenious method for studying the formation of virus inclusions *in situ*, working with the duck's egg infected with ectromelia and vaccinia. Briefly, the egg-shell was partly cut away and replaced by a coverslip, so that the membrane was visible. Microscopic examination was then carried out with a Heine Ultropak (Leitz), which makes use of the principle of annular oblique incidence illumination.

2. Cultivation of viruses.

A large number of viruses can be propagated by serial passage through eggs. Generally speaking, subculturing is carried out by grinding-up an infected membrane, and after centrifugation, injecting the supernatant fluid into a fresh egg. Animal or other titrations are usually carried out to prove that there is an actual increase in the virus content of the membrane.

The following human viruses have been propagated by serial passage through eggs: common cold (p. 526); influenza (p. 554); herpes (p. 161); Japanese encephalitis (p. 868); lymphocytic choriomeningitis (p. 898); lymphogranuloma inguinale (p. 187); measles (p. 221); psittacosis (p. 590); Rift Valley fever (p. 416); St. Louis encephalitis (p. 860); sand-fly fever (p. 511); varicella (p. 256); yellow fever (p. 487).

Numerous animal viruses have also been grown in the egg: ectromelia; equine encephalomyelitis; fowl-plague; fowl-pox; infectious laryngo-tracheitis of fowls; Kikuth's canary virus; louping-ill; Newcastle disease of fowls; Pacheco's parrot disease; vesicular stomatitis of horses.

3. Titration of viruses.

Virus titrations can, of course, be carried out on laboratory animals such as the rabbit. If so, one may use death of the animal as the determining factor in estimating the end-point, or else intradermal reactions may be studied. If the egg is substituted for the rabbit, likewise death of the embryo may be used as the end-point, or the number of pocks on the chorio-allantois may be counted, according to the virulence of the particular strain of virus used.

Much of this work has been carried out by Burnet and his collaborators, whose papers should be consulted for fuller details than can be given here. Thus Burnet and Ferry (1934), in their studies on fowl-plague and Newcastle disease, inoculated eggs with varying dilutions of virus, and found that infective doses produced death of the embryo within 48 hours. The method whereby the potency of the virus is estimated by counting the number of pocks produced on the chorio-allantois has been carried out for a number of viruses: vaccinia, by Keogh (1936); ectromelia, by Burnet and Lush (1936 *a*); Kikuth's canary virus and fowl-pox, by Burnet and Lush (1936 *b*); and infectious laryngo-tracheitis by Burnet (1936 *a*). As an illustrative example of the methods used in this class of work, the study of Keogh (1936) on vaccinia may be cited. Eggs incubated for 12 days were inoculated with virus, and pock-counts made after 48 hours. An initial series of experiments was carried out to determine the approxi-

mate strength of virus giving discrete pocks. Thereafter, falling dilutions of 3 were prepared, starting at 1/300 and continuing down to 1/24,300, the bulk of inoculum being 0·05 c.c. The 1/300 dilution was found to give too many pocks to count accurately, and the pocks in the 1/900 dilution were also somewhat confluent; the 1/24,300 dilution gave an average of 7·5 pocks, the 1/2,700 and 1/8,100 dilutions giving figures of 61 and 21 pocks. It was found by a parallel series of intradermal titrations in rabbits that the titres obtained by this method corresponded closely with those by the pock-counting method.

4. Isolation of viruses.

It has been claimed that influenza virus may be isolated by direct inoculation of eggs with nasopharyngeal secretion (see p. 555).

It has recently been shown that the chorio-allantois may be used to test the distribution of virus in experimental animals after various routes of inoculation. Thus, working with louping-ill virus, Burnet and Lush (1938) were able to detect the presence of virus in the olfactory bulbs after intraperitoneal injection. They recommended that a standard suspension of the tissue to be tested should be prepared, and a set volume injected into the egg. Titration was carried out by counting the pocks produced on the chorio-allantois of 12-day embryos after 44 hours' incubation.

5. Titration of antiviral sera.

Eggs may be used in lieu of experimental animals to test the virus-inactivating properties of immune sera. Thus Burnet and Galloway (1934), working with vesicular stomatitis, mixed equal parts of immune serum with falling dilutions of a collodion membrane filtrate of virus. After incubation at 15° to 20° C. for 30 minutes eggs were injected. The usual lethal action of this virus on the embryo was prevented in adequately neutralized specimens. The test proved more sensitive to the presence of unneutralized virus than did a parallel series of injections in the guinea-pig's pad.

When dealing with viruses which produce local pocks on the chorio-allantois, the antiviral properties of immune sera can be tested by their power of diminishing, or actually preventing, these lesions. Thus, Keogh (1936) found that antivaccinial sera could be titrated by the percentage reduction effected in the numbers of typical vaccinial pocks, a reduction of 90 per cent. being usually obtained. Burnet (1936 *b*) records a routine method for testing fowl sera for their content of laryngo-tracheitis antibodies: the sera to be tested are mixed with an equal volume of virus (usually diluted 1/10,000), and 1 hour later eggs are injected. Potent sera may completely inhibit the development of the usual lesions.

6. For the preparation of vaccine for human use.

Egg-cultured virus has been quite widely used for human vaccination (see p. 373), and here we describe the technical details of the preparation of such material, Goodpasture *et al.* (1935), for example, giving the following instructions:

(*a*) The egg membrane may originally be inoculated with either ordinary lymph, testicular virus, dermal lapine, or directly from the human vaccinial pustule.

(*b*) For the first 3 to 4 passages an incubation period of at least 72 hours is required, although after this period it may be reduced to 48 hours.

(*c*) At least six eggs should be inoculated at each subculture.

(*d*) When the membrane is removed from the egg, a small piece from the centre of the lesion should be excised and smears stained for elementary bodies.

(*e*) Approximately one-quarter of the lesion is inoculated into dextrose infusion broth and culture carried out. The remainder is placed in the refrigerator to freeze.

(*f*) For initiating the next subculture, lesions should be used which show the largest number of Paschen bodies.

(*g*) To maintain the potency of 'seed' virus, do not use inocula which have been stored for a considerable period.

(*h*) When titrated, culture virus diluted 1/1,000 should produce a confluent eruption on 90 to 100 per cent. of the inoculated area in every test.

(*i*) To prepare vaccine for human inoculation, four parts by volume of 50 per cent. sterile glycerol in saline are added to one part of pulp; pulp is prepared by grinding frozen membrane till finely divided.

Stevenson and Butler (1939) have carried out extensive experiments on the preparation of egg virus in a form that could be used for human inoculation. They give the following instructions for the preparation of vaccine from chorio-allantoic membranes (a dermal strain of virus was grown):

(*a*) Chorio-allantoic membranes are not used if the embryo is dead, or if the yolk is ruptured in the course of removal; this is to obviate the danger of contamination. The chorio-allantois is usually removed from the egg after 4 days' growth.

(*b*) Operating inside a special box, vaccinial plaques are removed from the membranes with sterile scissors.

(*c*) Five to ten plaques may be collected in one dish, weighed, and frozen in the cold-room overnight.

(*d*) The dish is brought to the laboratory surrounded by freezing mixture. The plaques are ground in a mortar, or a special apparatus, to a pulpy consistence.

(*e*) Material for passage is removed at this stage and stored in screw-topped bottles at $-7°$ C. to $-10°$ C.

(*f*) To prepare vaccine, add 4 c.c. of 50 per cent. neutralized glycerol in distilled water to each 1 gm. of pulp. Store in sterile ampoules, screw-topped bottles, or capillary tubes.

(*g*) Cultures for sterility should be carried out in broth, agar, and cooked-meat medium.

(*h*) Vaccine may safely be stored for 6 to 7 months.

7. Other applications of the egg in the study of virus immunity.

(*a*) *The theory of the antigen-antibody reaction.* Burnet, Keogh, and Lush (1937) have shown that the egg membrane can be used to furnish evidence on the theoretical problem of the antigen-antibody reaction. They have used pock-counting and virus-neutralization tests with influenza, louping-ill, vaccinia and other viruses, and by the study of such methods they have obtained much useful information regarding the phenomena of the interaction of antigen and antibody.

(*b*) *As a source of antigen.* Infected egg membrane has been used as a source of antigen for the complement-fixation reaction in influenza (see p. 89).

In conclusion, it is thus evident that the egg is becoming a very important adjunct to modern virus technique.

REFERENCES

BENGSTON, I. A., and DYER, R. E. (1935), *Publ. Hlth. Rep., Wash.*, **50**, 1489.

BURNET, F. M. (1933), *J. Path. Bact.*, **37**, 107.

—— (1936 *a*), *J. exp. Med.*, **63**, 685.

—— (1936 *b*), *Med. res. Counc. spec. Rep.*, no. 220, H.M. Stationery Office, London.

BURNET, F. M., and FERRY, J. D. (1934), *Brit. J. exp. Path.*, **15**, 56.

BURNET, F. M., and GALLOWAY, I. A. (1934), *Brit. J. exp. Path.*, **15**, 105.

BURNET, F. M., KEOGH, E. V., and LUSH, D. (1937), *Aust. J. exp. Biol. med. Sci.*, **15**, 233.

BURNET, F. M., and Lush, D. (1936 *a*), *J. Path. Bact.*, **43**, 105.

—— —— (1936 *b*), *Brit. J. exp. Path.*, **17**, 302.

—— —— (1938), *Aust. J. exp. Biol. med. Sci.*, **16**, 233.

D'AUNOY, R., and EVANS, F. L. (1937), *J. Path. Bact.*, **44**, 369.

GOLDSWORTHY, N. E., and MOPPETT, W. (1935), *J. Path. Bact.*, **41**, 529.

GOODPASTURE, E. W. (1938), *Amer. J. Hyg.*, **28**, 111.

GOODPASTURE E. W., BUDDINGH G. J., RICHARDSON L., and ANDERSON K. (1935), *Amer. J. Hyg.*, **21**, 319.

GOODPASTURE, E. W., WOODRUFF, A. M., and BUDDINGH, G. J. (1931), *Science*, **74**, 371.

—— —— —— (1932), *Amer. J. Path.*, **8**, 271.

HIMMELWEIT, F. (1938), *Brit. J. exp. Path.*, **19**, 108.

KEOGH, E. V. (1936), *J. Path. Bact.*, **43**, 441.

ROUS, P., and MURPHY, J. B. (1911), *J. Amer. med. Ass.*, **56**, 741.

STEVENSON, W. D. H., and BUTLER, G. G. (1939), *Rep. publ. Hlth. med. Subj., Lond.*, no. 87.

WOODRUFF, A. M., and GOODPASTURE, E. W. (1931), *Amer. J. Path.*, **7**, 209.

PART V.

Miscellaneous papers dealing with herpes labialis and with Hodgkin's disease. The contents of these publications are self-explanatory.

CASES OF HERPES LABIALIS FOLLOWING SULPHAPYRIDINE AND T.A.B.

SUBMITTED FOR PUBLICATION TO JOURNAL OF THE ROYAL ARMY MEDICAL CORPS.

HERPES LABIALIS AFTER SULPHAPYRIDINE AND T.A.B. THERAPY. AN INTERESTING PHENOMENON.

By

C. E. van Rooyen, Major, R.A.M.C.

Pathologist at a Military Hospital.

Although herpes labialis is clinically a harmless infection, the causal agent of the disease has been the subject of much research. The condition is not infrequently encountered among soldiers, and a Swiss regimental physician (Mayer, 1921) has described a spontaneous outbreak of 30 cases that affected a company of 70 troops who slept in one barrack room. Five of six officers attached to this same unit were likewise involved. This winter, among hospital patients we observed 33 cases and investigated these from the laboratory point of view. Two classes of persons suffering from herpetic eruptions were studied, namely, normal healthy nursing orderlies who developed it spontaneously, and a larger group comprising patients treated for gonorrhoea.

Before commencing our description of the clinical-pathological findings and animal inoculation results, we should like to present a summary of modern conceptions regarding the aetiology/

ogy and pathogenesis of herpes. The disease was first proved to be infective by Vidal (1873) and thereafter the classical researches of Levaditi, Doerr and others showed that the causal agent was a filterable virus, which was highly pathogenic to the rabbit and produced violent keratitis if implanted upon the scarified cornea of that animal, and this condition was sometimes followed by meningitis and death. Individual strains of virus differed in their neurotropic affinities for the rabbit, and consequently attention turned to herpes simplex virus as being a possible cause of encephalitis lethargica, and Levaditi and Harvier (1920) claimed to have isolated the virus from such cases. Unfortunately, independent confirmation of Levaditi's researches was lacking and the work fell into disfavour, but it may still prove one day that Levaditi's investigations were made in advance of his time. The clinical nomenclature employed for the designation of the various manifestations of herpetic infection in man is unsatisfactory. Thus, the usage of terms such as herpes simplex, febrilis, labialis, recurrent herpes, idiopathic and symptomatic herpes, indicates that the classification is inconsistent, and not based on anatomical, clinical, or aetiological grounds. For the convenience of readers overseas, the following account contains a summary of the facts concerning the clinical types of herpes and the aetiology of the condition. At the outset it should be understood that all forms of herpes affecting man, <u>other than herpes zoster</u>, are due to one and the same virus, which is pathogenic to the rabbit, whereas the virus of herpes zoster is a separate entity/

entity which is non-pathogenic to the rabbit and probably related to the virus of varicella. Both herpes and zoster viruses are particulate and visible organisms which occur in the form of minute virus elementary bodies, and Elford et al. (1933) have succeeded in measuring herpes simplex virus and estimated it to be 100-150 μ in diameter. Herpes virus also produces specific histological changes, and acidophilic intra-nuclear inclusion bodies have been described in rabbit corneal epithelium (Fuchs, 1933), and in the large epithelial cells present in human vesicle fluid (Da Fano, 1923).

Clinical Varieties of Herpes.

Herpetic infection may appear in a variety of forms:

Herpes febrilis is the name applied to herpes arising during the course of a pyrexial disease, such as pneumonia, malaria, influenza, and other febrile disorders. The usual situation is on the lip when the term "herpes labialis" is popularly used, but when other areas of the face are involved it is called herpes facialis. Aphthous stomatitis is another disease which has been proved to be due to the herpes virus (Dodd, Johnston, and Buddingh, 1938; see also Gottron, 1938; Burnet and Lush, 1939). Herpetic vesicles are seldom bigger than a large pin-head and develop on a reddened base, being usually filled with clear fluid, although suppuration may occur. The commonest site for vesicles is on the lips, inside and around the mouth, the cheeks, and the auricles.

Herpes simplex is the designation applied to herpetic eruptions/

eruptions unassociated with pyrexia, which may arise spontaneously, or secondarily to a variety of causes, such as after the injection of vaccines, milk and colloidal metals (see Fischer, 1927); or the ingestion of certain foodstuffs followed by gastric disorder.

Herpes genitalis. Herpetic eruptions are not uncommon on the genitals, being found on the glans or body of the penis, and in the female on the labia. It is frequently associated with syphilis (Cranston Low, 1939).

Herpes cornealis. Herpetic infection of the cornea may assume a variety of forms, of which the dendritic ulcer is the best known (see Duke-Elder, 1938). The inflammatory reaction may be severe and result in serious scarring of the cornea.

Recurrent herpes. Certain persons are very susceptible to herpes, and why this should be is not clear. It is possible that they carry the virus in their saliva or elsewhere in the body, and Burnet and Lush (1939) have suggested that in such instances the infection is contracted in infancy, as an aphthous stomatitis, and persists throughout life. Some particular recurring susceptibility of the tissues may allow the virus to exert its pathogenic action from time to time, and certainly recurring genital herpes is most apt to occur in cases of irritation due to chronic gleet and prostatitis (Avit-Scott, 1931). In women recurrent herpes is often associated with the menstrual periods (Blum, 1926).

Apart from the question of variations in the host as the predisposing factor in recurrent herpes, it is possible that the strain of virus itself may be one possessed of unusual tenacity. Thus/

Thus, Nicolau and Banciu (1924) inoculated a number of persons with strains of virus from cases of recurrent infection, and found that two of these persons themselves developed recurrent herpes.

Nervous manifestations. Although herpes is usually regarded as a mild local lesion, more serious manifestations may arise from time to time. For example French authors (e.g. Levaditi, 1923, 1926) describe herpès hovralgique de Mauriac, with symptoms of severe pains, crises of hyperaesthesia in the perineum, tenesmus, sphincter spasm, and sciatica, all preceding the appearance of the eruption. In less severe cases, herpes may be accompanied by pruritus, backache, and neuralgia. Doubtless these are due to the fact that the virus involves the corresponding ganglia, producing an inflammatory reaction (see Howard, 1935). Herpetic meningitis is a distinct clinical entity, with sudden onset, rapid course, favourable outlook, and a lymphocytosis in the cerebrospinal fluid (see Abiteboul, 1936).

The Common Aetiology of Herpetic Eruptions.

To-day all herpetic eruptions (always excepting, of course, those of zoster) are regarded as due to one and the same virus, based on the following evidence:

Vesicle fluid from all varieties of herpetic eruption can be inoculated into the human skin with consequent production of a typical crop of 'simplex' vesicles (Vidal, 1873; Lipschütz, 1921; Teissier et al., 1922 b; Frei, 1931; Zurukzoglu and Hruszek, 1933).

Vesicle/

Vesicle fluid from herpes febrilis produces a severe keratitis when applied to the rabbit's eye (Löwenstein, 1919, 1920; Grüter, 1920).

Herpes cornealis yields a virus with the same characteristic effect (Doerr, 1920; Doerr and Vöchting, 1920; Luger and Lauda, 1921).

Herpes labialis yields the same virus, and will produce typical herpes cornealis when suitably applied to the human cornea (Doerr, 1920; Doerr and Vöchting, 1920; Fuchs, 1921, and see 1933).

Vesicle fluid from herpes genitalis also produces keratitis in the rabbit, and man can be reinfected with material taken therefrom (Fontana, 1921; Blanc and Caminopetros, 1921).

Herpes occurring in such diseases as cerebrospinal meningitis, pneumonia, diphtheria, influenza, catarrhal jaundice, erythemata, and secondary syphilis yields a virus with the same characteristics as that obtained from cases of primary herpes (Teissier et al., 1922 a).

Similar intranuclear inclusion bodies have been reported in human herpes febrilis, cornealis, and genitalis, and in experimental lesions in animals (Lipschütz, 1921 a,b,c; Fuchs, see 1933).

The Distribution of Virus in Infected and Healthy Persons.

Herpetic vesicle. The virus is abundantly present in the vesicular/

vesicular stage, but disappears after the crust forms (Teissier, Gastinel, and Reilly, 1926 a).

Blood. The virus may be found in the blood of persons suffering from herpetic eruptions, although this is rare (Löwenstein, 1919; Bastai and Busacca, 1924 b, Teissier, Gastinel and Reilly, 1926 a, b).

Cerebrospinal fluid. Flexner and Amoss (1925 a) examined the cerebrospinal fluid of 100 persons, including some suffering from epidemic encephalitis, but in only one case were they able to isolate the herpes virus, and this from a syphilitic patient with no herpetic eruption. This is an important observation and indicates that the virus may, although rarely, be found in the cerebrospinal fluid of patients who, at the moment, do not suffer from herpes. Zurukzoglu (1937) has also isolated two strains of herpes virus from the cerebrospinal fluid of healthy persons.

With regard to the cerebrospinal fluid of herpetic patients, the consensus of opinion is that the virus may be found on occasions (Revaut and Rabeau, 1921; Bastai and Busacca, 1924 a; Zurukzoglu, 1937). It should be added, however, that a number of competent investigators have been unable to detect its presence (e.g. Doerr and Zdansky, 1924; Levaditi, 1925; Teissier, Gastinel and Reilly, 1926 a, b).

Saliva. Herpes virus has been found in the healthy saliva by a number of workers (e.g. Levaditi, Harvier, and Nicolau, 1921; Doerr and Schnabel, 1921; Teissier, Gastinel, and Reilly, 1926 b). The/

The virus is attached to the salivary epithelial cells and is probably associated with the buccal mucus, because suspensions of salivary gland tissue are avirulent (Levaditi, Harvier, and Nicolau, 1921). The presence of the virus has usually been detected by its power of producing keratitis in rabbits. Levaditi and his collaborators have stated that 80 per cent. of normal specimens of saliva produce experimental keratitis, but only in 15 per cent is this keratitis severe enough to be followed by cerebral involvement (vide infra). These high figures have not, however, been generally accepted by other workers, who have failed to find any virus in normal saliva (e.g. Flexner and Amoss, 1925 c). Nevertheless there seems no doubt that the virus can on occasions be found in this site as we will describe below.

Several workers have investigated the saliva of persons suffering from herpes and have, though rarely, been successful in demonstrating the presence of the virus (e.g. Isaicu and Telia, 1922; Flexner and Amoss, 1925 b; Nicolau and Poincloux, 1922, 1924). Thus, it has been found in the saliva of patients suffering from labial and facial herpes and herpes of the finger. The saliva of persons predisposed to herpetic eruptions contains the virus more frequently than that of other persons, and it may be found in the intervals between attacks (Levaditi, 1926).

Conjunctival sac. The virus can apparently be carried in a latent form in the conjunctival sac, but if the cornea be injured, then it may produce keratitis (Grüter, 1924; Busacca, 1925). It may also cause severe unilateral conjunctivitis, developing without obvious cause. (Granström, 1937).

Other skin lesions. The virus of herpes has been recovered on rare occasions from zoster vesicles and sycosis pustules (Naegeli and Burukzoglu, 1935).

Our present article deals with the condition of herpes labialis which developed in healthy (often perfectly fit) male adults or, as in the majority of our cases, in patients under treatment for gonorrhoea. Below we describe our own work which is subdivided into clinical data, laboratory findings, animal inoculation experiments (performed by Dr. Rhodes), followed by a discussion on the aetiology of the condition.

Clinical Data.

Altogether 33 cases of herpes labialis were observed by us, 6 occurred spontaneously in healthy Nursing Orderlies, and 27 in patients suffering from gonorrhoea who had received a full course of sulphapyridine and subsequently developed herpes labialis after a provocative dose of T.A.B. The dosage of sulphapyridine administered was that employed prior to the latest Army Instructions, and consisted of 9 gms. on the first day; 6 on the second and third; and 6 on the fourth, the total quantity given being 25 gms. in four days. The clinical results of such therapy were excellent and in the majority of instances the urethral discharge had abated by the fifth day, so that on the sixth day patients underwent a test of cure consisting of a 0.2 cc. dose of T.A.B. vaccine injected intravenously. It was following this provocative dose of T.A.B. that herpes frequently manifested itself. In the first batch/

batch of observations, twenty two patients received 0.2 cc. of T.A.B. vaccine intravenously and 48 hours later it was noticed that all the men developed herpes labialis. Sixteen showed only a slight eruption which cleared up spontaneously without any treatment, but in the remaining six, the lesions were severe and a 50% absolute alcohol dressing was applied with good effects.

Following these results it was decided by Capt. J. A. Scott, R.A.M.C., to reduce the dose of vaccine and the next batch of 6 cases received 0.1 cc. T.A.B. intravenously. Once again herpes was observed, and three persons were affected. Subsequently, it was decided by Capt. Cameron Ewing, R.A.M.C., to dispense with intravenous T.A.B. as a provocative measure and thereafter hypertonic saline solution (instilled and retained in the urethra fur a short time) was substituted as an alternative, following which no further cases of herpes appeared. The obvious conclusion to be drawn from these results is that if herpes labialis is to be avoided, intravenous T.A.B. vaccine should not be given to persons immediately after they have received a course of sulphapyridine. This is particularly so during the winter months when, as we shall mention later, the herpes carrier rate is relatively high.

To provide a reason for this curious phenomenon is not easy, and at first sight it seemed that the T.A.B. was the primary exciting cause since it is an acknowledged fact that the parenteral injection/

injection of bacterial protein, such as T.A.B., gonococcus and others may sometimes cause herpes labialis in a small proportion of individuals. But we had reason to suspect that the predisposition to herpetic infection was greatly enhanced by the intake of sulphapyridine, because no less than 24 out of 25 patients who received 0.2 cc. of T.A.B. intravenously and 3 out of 6 receiving 0.1 cc. of T.A.B. after sulphapyridine, developed herpes. Thus, we have inferred that whilst a course of sulphapyridine does not provoke herpes itself, the drug increases the susceptibility to herpes if a subsequent and large enough dose of T.A.B. is given intravenously. We need hardly add that for obvious reasons one cannot answer the question of how many patients would have developed herpes if they had received intravenously T.A.B. without sulphapyridine.

The possibility of the batch of T.A.B. vaccine used having become accidently contaminated with herpes virus was explored, but animal inoculation and sterility tests proved that the product was faultless. Finally one of us (C. E. v. R.) received 1 cc. of this same vaccine subcutaneously without developing herpes.

Laboratory Investigations.

Animal experiments.

Herpes virus was recovered from six out of seven cases examined by employing the following technique:- Vesicle fluid was collected in sterile capillary glass tubes; a drop of the fluid was placed on the corneal surface of an anaesthetised rabbit and the/

the surface of the organ lightly scarified with a needle. After 48 hours acute kerato-conjunctivitis had developed. A proportion of these animals developed meningitis and died, and the condition could be transmitted to healthy rabbits by inoculating them intra-cerebrally or corneally, with infected brain or corneal tissue suspensions.

We also demonstrated the presence of elementary bodies of herpes in films prepared from the conjunctival exudate and from corneal scrapings of infected rabbits. Likewise dark back-ground microscopical examination confirmed that numerous highly refractile particles not unlike elementary bodies could be seen, but much reliance was not placed on this method of examination for reasons already discussed by van Rooyen and Rhodes (1940).

Examination of T.A.B. vaccine.

The phial of material used for provocative injection was carefully examined for possible evidence of contamination with herpes virus, but although rabbits were inoculated by various routes, none could be recovered. Simultaneously, general tests for absence of bacterial growth were conducted and 1 cc. amounts of bacillary emulsion were inoculated in suitable solid and fluid media and incubated aerobically and anaerobically in a Mackintosh and Fildes jar at 37°C. for 14 days, but as previously, results were negative throughout. No blame could therefore be attached to the T.A.B. vaccine.

Leucocyte/

Leucocyte counts on patients.

These were done for two reasons, first to keep a check on the white cell level during sulphapyridine treatment and secondly, to ascertain whether herpes occurred more frequently in patients possessing a low polymorph count. Results showed that no correlation existed between the degree of leucopenia and the onset of herpes, for in some cases after sulphapyridine the w.b.c. fell to 6000 per cu.mm., whereas in others it remained as high as 9000, but nevertheless, both categories developed herpes labialis after a provocative dose of T.A.B. Likewise, twelve additional patients possessing white cell count figures intermediate to the above also came under review, but again herpes developed indiscriminately. Finally, no relationship could be shown to exist between the state of the differential white cell picture and the predisposition to herpes.

Carriers of Herpes Virus.

It is universally accepted that herpes virus may occur as a commensal in the saliva of a large proportion of normal healthy human beings. According to Andrewes and Carmichael (1930) specific serum virus-neutralizing antibodies were discovered in 75% of individuals at the time of their experiments. What percentage of normal persons carry herpes virus it is difficult to calculate, but it is certain that the figure would vary from time to time and would most probably be highest among those living in camps/

camps and hospitals, especially during the winter months. To obtain a rough estimate of the carrier rate in a single ward in a hospital during December 1940, we collected early morning specimens of saliva from ten cured patients awaiting discharge to duty, and examined them for evidence of herpes virus. Nine of these specimens of saliva contained no virus, but from the tenth a typical strain of herpes was recovered and passaged serially through three rabbits. Owing to the high cost of animals a larger number of tests could not be performed. The presence of herpes virus in one out of the ten persons examined illustrates how easily it can be demonstrated in the mouths of normal individuals, and at a guess something like 10% of persons could therefore be assumed to be carriers of virus in this institution at this time of the year. It shall be remembered that every carrier of the virus does not necessarily show herpetic lesions, but on the other hand, the converse is true, for every patient who develops herpes is proof of the presence of the virus in that individual's saliva. Thus, it is quite feasible for a high virus carrier rate to exist among a community who may exhibit a relatively low case incidence of herpes labialis, unless methods of therapy which are known to exert a herpetogenic action are used. In support of this hypothesis we would point out that during the winter months we noticed six cases of herpes which occurred spontaneously among healthy nursing orderlies and others in a unit of about 300 strong. But when patients were treated with sulphapyridine and 0.2 cc. intravenous/

intravenous T.A.B., no less than 24 out of 25 developed herpes, thus proving the high carrier rate existent.

Repeated observations three months later.

From December to March intraurethral saline was substituted for T.A.B. vaccine as a provocative measure, and over a hundred cases of gonorrhoea were effectively treated with sulphapyridine without any sign of herpes. At the end of this interval, three cases of chronic gonorrhoea which had responded unfavourably to sulphapyridine arrived for attention, and it was decided to try the effect of mild protein shock therapy. Accordingly, each patient received intravenously 0.2 cc. from a fresh phial of T.A.B., and five days later two of the men developed herpes labialis. These results are of particular significance since they support the principal points we had established earlier, namely, that the 25 cases already observed were not due to a spontaneous outbreak; and although virus was still prevalent in the saliva of most patients, the use of sulphapyridine alone did not provoke herpes, but no sooner was intravenous T.A.B. used, herpes appeared.

DISCUSSION.

Clinically, herpes labialis is such a trivial affection that it scarcely seems to warrant much attention, but there is reason to suggest that the virus is responsible either in part or in whole for certain other pathological lesions in man, (see Levaditi and Lepine, 1940) and consequently, we believe that continued/

tinued study of herpes may yield valuable information. Certain facts have long been recognised, for instance that it is frequently present in human saliva, and is liable to attack healthy persons without the slightest provocation. More often however, it is customary to meet it in those whose health is poor; have suffered from an acute febrile illness such as an attack of influenza or pneumonia, or whose vitality has been temporarily diminished by some form of treatment such as pyrotherapy or protein shock.

Warren et al. (1940), reported that symptomatic herpes developed in 190 (46%) of 411 persons treated with fever induced by methods such as radiothermy (30 metres), infra-red radiations with carbon filament lamps and hot water baths. The temperature employed being 39.5 to 41.5°C. for periods ranging from 1 to 47 hours. Several important points were established during the conduct of their researches, for instance, it was not possible to link the frequency of herpes with the height of pyrexia attained, the duration of its application or the method employed for its generation. The only relationship which could be adduced was the fact that the incidence of herpes seemed to be maximum during seasons of the year when naso-pharyngeal infections were most prevalent. Thus during the month of November, 57 fever treatments were administered and 50% developed herpes, whilst in the summer month of June, 32 patients received treatment and only 12% contracted herpes. Cases seemed to occur in series, and sometimes for/

for intervals of 10 to 30 days none appeared, but at others, every patient who was treated within a period of a week or longer developed it.

Warren and his associates have not offered any explanation for the latter phenomen, but it is likely that variation in the normal herpes carrier rate was the most likely reason as to why cases were more numerous at certain times than at others. Under normal circumstances herpes labialis tends to be a self-limiting process which heals readily without complications, but the careful observations of Warren et al. have disclosed that this is not invariably so, since some of their patients revealed signs and symptoms of meningo-encephalitis resembling a condition named herpetic fever, described by Plessing (1929). In the present article we have adduced further evidence in support of two well established facts concerning the pathogenesis of herpes. First that it is to be frequently found in apparently normal human saliva, and secondly, that following protein shock therapy herpes labialis may develop. Two new points are also mentioned, namely, that large doses of sulphapyridine sufficient to cure gonorrhoea fail to abolish herpes virus in the saliva of carriers, and lastly, that after a course of sulphapyridine, pyrotherapy or protein shock is more likely to provoke herpes labialis.

At this stage the reader may well ask why should a course of M & B 693 render an individual liable to an attack of herpes? Unfortunately we can offer no explanation as to why this should be/

be so. It is conceivable, however, that the phenomenon may resemble an effect opposite to that of non-specific stimulation of antibody formation by the injection of certain chemical salts, e.g., beryllium chloride, manganous chloride, colloidal manganese, salvarsan (Browning and Mackie, 1937). According to Nicolau (1938) benzol, arsenic, arsenobenzol, iodine, mercury, alcohol and cocaine also have a tendency to be herpetogenic, and consequently sulphapyridine is unique since it does not cause herpes itself, but enhances the herpetogenic effect of T.A.B. It might be that sulphapyridine depresses the natural antibody level of human blood to herpes virus (see Brain 1937), so that to a carrier a subsequent mild protein shock precipitates an attack, but this fails to explain everything.

In conclusion, we feel that there is something to be learnt in connection with our experiences, and the obvious lesson is that sulphapyridine ought not to be used for treating herpes labialis or any other disease caused by herpes virus. Unfortunately, very little is understood of the pathogenesis of herpes virus and although certain workers have claimed that such diseases as encephalitis lethargica and the dendritic ulcer of the cornea are manifestations of herpetic infection, the whole question of the pathogenicity of herpes virus requires re-investigation in the light of modern knowledge of virus infections.

CONCLUSIONS/

CONCLUSIONS.

1. Twenty seven out of thirty one patients developed herpes labialis after a course of sulphapyridine followed by an intravenous injection of 0.2-1 cc. of T.A.B. emulsion.
2. All these persons were probably salivary carriers of the herpes virus.
3. Large doses of sulphapyridine (25 gms. in 4 days) do not destroy herpes virus in human saliva, neither do they cause herpes labialis in carriers.
4. Although sulphapyridine does not provoke herpes, the drug appears greatly to enhance the herpetogenic effect of T.A.B. vaccine if administered intravenously.
5. The phenomenon we have described is not due to leucopenia and we do not understand why it should occur.

ACKNOWLEDGMENTS.

I desire to thank Col. J. M. Mackinnon of No. 33 General Hospital for permission to publish the data recorded, and wish to thank Lt. Col. G. R. MacNab and Capt. J. A. Scott, R.A.M.C., for their interest.

REFERENCES

REFERENCES.

Abiteboul, J. (1936), Thèse de Paris, No. 599.

Andrewes, Ch., and Carmichael, E. A. (1930), Lancet, 1, 857.

Avit-Scott, J. (1931), Lancet, 1, 972.

Bastai, P., and Busacca, A. (1924 a), Münch. med. Wschr., 71, 1056.

Bastai, P., and Busacca, A. (1924 b), Klin. Wschr., 3, 442.

Blanc, G., and Caminopetros, J. (1921), C. R. Soc. Biol., 84, 859.

Blum, R. (1926), Zbl. Gynäk., 50, 540.

Brain, R. T., (1937), Brit. med. J., 2, 1064.

Browning, C. H., and Mackie, T. J. (1937), Muir and Ritchie's Manual of Bacteriology, Oxford University Press.

Burnet, F. M., an Lush, D. (1939), Lancet, 1, 629.

Busacca, A. (1925), Arch. Augenheilk., 95, 253.

Cranston Low, R. (1939), Diseases of the skin, Oliver and Boyd, Edinburgh.

Da Fano, C. (1923), J. Path. Bact., 26, 85.

Dodd, K., Johnston, L. M., and Buddingh, G. J. (1938), J. Pediat., 12, 95.

Doerr, R. (1920), Klin. Mbl. Augenheilk., 65, 104.

Doerr, R., and Schnabel, A. (1921), Schweiz. med. Wschr., 51, 469, 562.

Doerr, R., and Vöchting, R. (1920), Rev. gén. Ophtal., Paris, 34, 409.

Doerr, R., and Zdansky, E. (1924), Z. Hyg., 102, 1.

Duke-Elder, Sir W. S. (1938), Text-Book of ophthalmology, Henry Kimpton, London.

Elford/

Elford, W. J., Perdrau, J. R., and Smith, W. (1933), J. Path. Bact., 36, 49.

Fischer, M. (1927), Z. Hyg. InfektKr., 107, 102.

Flexner, S., and Amoss, H. L. (1925 a), J. exp. Med., 41, 215.

Flexner, S., and Amoss, H. L. (1925 b), J. exp. Med., 41, 357.

Fontana, A. (1921), Pathologica, 13, 406.

Frei, W. (1931), Derm. Wschr., 93, 1508.

Fuchs, A. (1921), Wien. klin. Wschr., 34, 307.

Fuchs, A. (1933), Brit. J. Ophthal., 17, 193.

Gottron, H. (1938), Mschr. Kinderheilk., 74, 82.

Granström, H. O. (1937), Acta ophthal., Kbh., 15, 361.

Grüter, W. (1920), Klin. Mbl. Augenheilk., 65, 398.

Grüter, W. (1924), Münch. med. Wschr., 31, 1058.

Howard, W. T. (1905), Amer. J. Med. Sci., 130, 1012.

Isaicu, L., and Telia, L. (1922), C. R. Soc. Biol., Paris, 87, 57.

Levaditi, C. (1922), Ectodermoses Neurotropes (Poliomyélite, encéphalite, herpès), Masson et Cie, Paris.

Levaditi, C. (1925), Paris méd., 1, 573.

Levaditi, C. (1926), L'Herpès et le Zona, Masson et Cie, Paris.

Levaditi, C., and Harvier, P. (1920), C. R. Soc. Biol., Paris, 83, 354.

Levaditi, C., Harvier, P., and Nicolau, S. (1921), C. R. Soc. Biol., Paris, 84, 817.

Levaditi, C., and Lépine, P. (1940), Les Ultravirus des Maladies humaines, Maloine, Paris. 298.

Lipschütz, B. (1921 a), Wien. med. Wschr., 71, 231.

Lipschütz/

Lipschütz, B. (1921 b), Derm. Wschr., 73, 798, 983.

Lipschütz, B. (1921 c), Arch. Derm. Syph., Wien, 136, 428.

Löwenstein, A. (1919), Münch. med. Wschr., 66, 769.

Löwenstein, A. (1920), Klin. Mbl. Augenheilk., 64, 15.

Luger, A., and Lauda, E. (1921), Wien. klin. Wschr., 34, 132.

Mayer, K. (1921), Schweiz. med. Wschr., 30, 703.

Naegeli, O., and Burukzoglu, S. (1935), Zbl. Bakt., Abt. I, Orig., 135, 297.

Nicolau, S. (1938), Les Ultravirus, Maloine Paris, 298.

Nicolau, S., and Banciu, A. (1924), C. R. Soc. Biol., Paris, 90, 138.

Nicolau, S., and Poincloux, P. (1922), C. R. Soc. Biol., Paris, 87, 451.

Nicolau, S., and Poincloux, P. (1924), Ann. Inst. Pasteur, 38, 977.

Plessing, (1928), See Warren et al. 1940.

Ravaut and Rabeau (1921), C. R. Soc. Biol., Paris, 85, 1132.

Teissier, P., Gastinel, P., and Reilly, J. (1922 a), C. R. Soc. Biol., Paris, 86, 73.

Teissier, P., Gastinel, P., and Reilly, J. (1922 b), C. R. Soc. Biol., Paris, 87, 648.

Teissier, P., Gastinel, P., and Reilly, J. (1926 a), C. R. Soc. Biol., Paris, 94, 377.

Teissier, P., Gastinel, P., and Reilly, J. (1926 b), J. Physiol. Path. gén. 24, 301.

van Rooyen/

Van Rooyen, C. E., and Rhodes, A. J. (1940), Virus diseases of man, Oxford University Press.

Vidal, J. B. (1873), Ann. Derm. Syph., Paris, 4, 350.

Warren, S. L., Carpenter, C. M., and Boak, Ruth A. (1940), J. exp. Med., 71, 155.

Zurukzoglu, S. (1937), Zbl. Bakt., Abt. I, Orig. 139, 86.

Zurukzoglu, S., and Hruszek, H. (1933), Zbl. Bakt., Abt. I, Orig., 128, 1.

The Interpretation and Significance of Gordon's Test in the Diagnosis of Hodgkin's Disease

A Study of 100 Cases

BY

C. E. VAN ROOYEN, M.D.

(*From the Department of Bacteriology, Edinburgh University*)

Reprinted from the EDINBURGH MEDICAL JOURNAL, N.S. (IVth), VOL. XLIV., p. 455, 1937

THE INTERPRETATION AND SIGNIFICANCE OF GORDON'S TEST IN THE DIAGNOSIS OF HODGKIN'S DISEASE.*

A Study of 100 Cases.

By C. E. VAN ROOYEN, M.D.

(*From the Department of Bacteriology, Edinburgh University.*)

THE aim of this article is an endeavour to present to the reader a concise account of recent experimental work on lymphadenoma. Gordon (1932) demonstrated that the intracerebral inoculation of a rabbit with an emulsion of lymphadenomatous lymphatic tissue was followed by paralysis, ataxia, musculo-incoordination and sometimes death of the animal. His discovery has been utilised to form the basis of a biological test for the diagnosis of Hodgkin's disease by van Rooyen (1933) and Ogilvie and van Rooyen (1933-34), who have drawn attention to the value of this procedure to the clinician and pathologist alike.

Their findings have received the confirmation of several independent workers, such as van der Hoeden and Hulst (1934), Davidson (1934), Paterni, Maroncelli and Corsi (1935), Davis (1935), Smith (1935), Bartolozzi (1934), Rosenberg and Bloch (1936), Uhlenhuth and Wurm (1936) and Warner (1936), to whose publications reference should be made for further details concerning the practical utility of Gordon's test.

Whereas the test is useful as an aid to the routine histological examination of gland tissue derived from suspected cases of Hodgkin's disease, much dubiety has existed with regard to the exact nature of the pathogenic agent present in this condition and responsible for the encephalitic syndrome in the rabbit. Considerable light has been shed on this problem by the work of Friedemann (1934), who showed that normal leucocytes, bone-marrow and splenic tissue may also exhibit an encephalitogenic property towards the rabbit similar to that produced by lymphadenomatous lymphatic glands. Friedemann (1934) further suggested that this agent is Jochmann's proteolytic enzyme, and demonstrated its ability to withstand the prolonged action of alcohol, ether and glycerol.

* By aid of grants received from the Science Committee of the British Medical Association and the Medical Research Council.

MacKenzie and van Rooyen (1935) have confirmed Friedemann's findings as regards the distribution of the pathogenic agent in human bone-marrow, spleen and leucocytes and have drawn the following conclusions from their studies: (1) the encephalitogenic principle present in normal tissues (*vide supra*) is indistinguishable from Gordon's agent observed in lymphatic glands affected with lymphadenoma; (2) Gordon's agent cannot be identified with Jochmann's proteolytic enzyme since no relationship can be found to exist between the proteolytic activity of a specimen of tissue examined on the one hand and its encephalitogenic properties on the other. Both investigators have agreed, however, that the pathogenic agent represents a product associated with certain normal and pathological cells.

On account of the resemblance existing between the encephalitic syndrome in the rabbit produced by lymphadenomatous tissue and that produced by certain neurotropic viruses, the possible virus nature of the pathogenic agent has been investigated (van Rooyen, 1934). Efforts to transmit the encephalitic condition from one rabbit to another have failed, and those animals which recovered from it were found to be again susceptible on re-inoculation. No definite immunity phenomena have been demonstrable and a search for inclusion bodies in the central nervous system of affected rabbits has failed to reveal them.

On the other hand, Gordon (1932) and Coles (1934) have reported the presence of minute particles resembling the elementary bodies of vaccinia in glands affected with Hodgkin's disease. It is difficult, however, to accept their presence alone as conclusive evidence for the assumption that lymphadenoma is a virus disease, especially when considered along with certain noteworthy negative findings, such as those already referred to above. There is no alternative, therefore, but to conclude that Gordon's agent represents a tissue derivative of certain normal and pathological cells which exhibits a toxic effect towards the central nervous system of the rabbit.

With regard to the distribution of the encephalitogenic agent in animal tissues other than those of the human subject, Friedemann (1934) has drawn attention to its occurrence in the bone-marrow of monkeys. This question is under investigation and will be dealt with elsewhere.

The Limitations of Histological Evidence as a Means for the Diagnosis of Hodgkin's Disease.

When the histopathological appearance presented by biopsy or necropsy material are typical of Hodgkin's disease in a suspected case of this condition, the diagnosis may be readily established. In an attempt to elicit the maximum information from a given case and obviate the chance of securing an enlarged gland which is inadequately representative of the nature of the pathological process as a whole, both Cunningham (1915), and Barron (1926), have advocated the simultaneous resection and examination of several glands at one time.

Unfortunately, however, there remains a proportion of instances in which no definite conclusions can be drawn from the results of microscopical examination. This is in accordance with the experience of numerous morbid histologists, for the existence of a group of conditions closely resembling but not identical with classical Hodgkin's disease has long been recognised. Thus, after an extensive review of the subject dealing with the histological diagnosis of Hodgkin's disease, Wallhauser (1933) states : " The criteria necessary to establish a diagnosis of Hodgkin's disease are hardly standardised or uniform, and the apparently increasing inclination of some writers to submerge all or many neoplastic or neoplasmoid structures of lymphoid origin under the enveloping and unqualified term of lymphoblastoma has further complicated the problem." He continues to quote Sternberg, who records that " The diagnosis of a typical lymphogranuloma has come to mean about as much as pseudoleukæmia, into which everything was placed that did not fit elsewhere. This is a pitiable back-step." Likewise, Kettle (see Pullinger, 1932) has suggested the term " Hodgkin group " as a suitable designation in order to include those cases which presented atypical characteristics.

In a paper dealing with the diagnosis of lymphadenoma, Ritchie (1935) advocated the routine performance of Gordon's test in all suspected cases of Hodgkin's disease. He further remarks that " The histological findings are often difficult of interpretation and depart very widely from the classical description of Reed and others, whilst blood examinations are often of more value in excluding the diagnosis than in confirming it. Nevertheless, amongst the ill-defined group

of progressive enlargements of the lymph glands there are certain cases concerning which the clinician, the pathologist and hæmatologist can reach ultimate unanimity, even if this happy issue is reached only over the autopsy table." Calvert and Sanguinetti (1933) also draw attention to the extraordinary difficulties which may be sometimes met with in the diagnosis of Hodgkin's disease.

Remarks such as these indicate the unsatisfactory state of our knowledge concerning the nature of hyperplastic conditions affecting the lymphoid and the reticulo-endothelial systems generally. They furthermore illustrate that notwithstanding the multiplicity of available dyes employed for staining lymphoid structure, the total data gleaned from microscopic examinations of such tissue still leaves much to be desired.

It must be appreciated that the morbid-histologist is only able to observe the staining properties, the shape, size, contents and configuration of particular cells, or groups of cells, from which deductions have to be made as to the possible nature of the pathological process at work. In lymphadenoma, owing to the fact that a multiplicity of cell types are probably derived from the reticulum of the gland, it is only to be anticipated that intermediate and ill-defined groups of cells are to be encountered in a certain percentage of cases, thereby complicating the histological picture.

The property of lymphadenomatous tissue to produce an encephalitic syndrome in the rabbit must accordingly be looked upon as an important characteristic. It will be shown later in this paper that Gordon's test is closely associated with the reticulum cell itself, and as such indicates a particular biological property of this type of cell apart from its appearance in stained sections.

The Interpretation of the Biological Test.

Merely because a similar syndrome to that produced by lymphadenomatous glands may also be elicited by the intracerebral inoculation of bone-marrow and splenic tissue, it must not be concluded that the value of the test is lessened. It should be clearly understood that Gordon's biological test for Hodgkin's disease only refers to the results following the intracerebral inoculation of rabbits with enlarged lymphatic glands and is not applicable to the effects of any other tissues when injected by the same route.

Gordon's Test in Diagnosis of Hodgkin's Disease

During the course of this work, extending over a period of four years, biopsies have been performed on 100 cases of suspected Hodgkin's disease and the tissue examined both histologically as well as subjected to biological test. Comparison of findings has revealed that the test is negative in tuberculosis, lymphosarcoma, lymphatic leukæmia, syphilis and pseudo-leukæmia (or a-leukæmic leukæmia), but positive in a high percentage of cases of lymphadenoma. It may, therefore, be presumed that Hodgkin's disease is the commonest cause of lymphadenohypertrophy that yields a positive result in this test.

Exceptions to this statement have been few in number: in one case a positive result was obtained in a possible case of Hodgkin's disease complicated by glandular tuberculosis. In another, a similar finding was observed in a boy aged 11, who clinically presented the text-book description of lymphadenoma but, on histological examination, displayed a marked eosinophilia without any other features sufficiently characteristic to warrant a diagnosis being pronounced.

Exceptional instances such as these are only to be expected when dealing with a laboratory method of diagnosis, and it may therefore be safely said that the percentage of error associated with Gordon's test compares favourably with those of other procedures involving the use of biological reagents in routine diagnosis. The test applied to lymphatic glands yields information that is of assistance to the morbid-histologist in the interpretation of histological results in suspected cases of lymphadenoma, especially when doubt exists with regard to the appearances seen in sections. A positive result in circumstances such as these should be regarded as evidence in favour of Hodgkin's disease. (*See* Ogilvie and van Rooyen, 1934.)

A negative result, however, is of limited significance and may sometimes be encountered in typical cases of lymphadenoma. The test should be repeated in such a case for it has been noticed that individual specimens of lymph nodes have been found to vary in their encephalitogenic activity. For example, in an earlier case of lymphadenoma it was found that whilst a small cervical gland removed at biopsy gave a negative test, after death of the patient, glands removed from the mediastinum and groin gave a positive reaction.

Gordon's biological test is positive in 85 per cent. of cases of lymphadenoma (Gordon, 1934), and positive in 75 per cent.

and 69 per cent. of cases according to van Rooyen (1934) and Davis (1935) respectively. It will thus be observed that the test has been found to be negative in a certain percentage of cases which were both clinically and histologically characteristic of Hodgkin's disease.

Furthermore, it should be pointed out that great variability exists with regard to the degree of encephalitogenic action displayed by material derived from different cases of Hodgkin's disease. For example, whereas one specimen may cause marked ataxia, paralysis, and death within three days, another may produce milder lesions from which the animal may recover completely or be entirely unaffected by it. Attention has been paid to this aspect of the problem and, accordingly, glandular tissue has been examined microscopically with a view to determining whether the variability in encephalitogenic behaviour may be explained on a basis of cellular composition.

It was previously reported (van Rooyen, 1933) that those lymph glands which were tough and fibrous in consistency frequently exhibited little or no encephalitogenic activity towards the rabbit and thus gave a negative result. Further work has shown that, to some extent, the result of the test may be influenced by the amount of fibrosis present in any particular specimen examined. This feature was illustrated in the case of two specimens removed from a single case of lymphadenoma, the one obtained at biopsy which gave a negative result, and the other at necropsy that yielded a positive (*vide supra*). Comparison of sections in such a case revealed that whereas the former contained excessive fibrous tissue in its composition, the latter displayed a greater degree of cellularity in structure. There has thus been some evidence to indicate that the presence or absence of the test is related to the degree of cellularity manifested by various specimens of glands and therefore explanation has been sought to interpret the reaction on a basis of such findings.

The Relationship of Gordon's Reaction to Histological Structure in Lymphadenoma.

The microscopic anatomy of typical Hodgkin's disease has been fully described by several observers such as Greenfield (1878), Andrewes (1902), and others; no further account is therefore necessary. The author would remind the reader, however, that the usual sequence of events occurring in

lymphatic glands are essentially, initial hyperplasia of lymphoid tissue, followed by the appearance of numerous endothelial cells, with a corresponding diminution in the numbers of existing lymphocytes.

These reticulo-endothelial cells are large in size, oval or fusiform in shape, contain a pale staining nucleus of vesicular type, showing chromatin granules within it and surrounded by a distinct nuclear membrane. Considerably larger cells, called Hodgkin giant cells, are also observed at this stage, and these are recognisable by virtue of their comparatively large size and characteristic multiple or convoluted nuclei. Eosinophilic polymorphonuclear leucocytes are frequently present and neutrophilic leucocytes are sometimes seen in addition. As the condition progresses the normal glandular architecture tends to be obliterated and replaced by the elements described above. The gland capsule is usually unaffected by these changes. Connective tissue fibres eventually make their appearance, and wide areas of fibrosis may be seen throughout the section.

Hitherto, attempts to correlate the occurrence of a positive reaction in the rabbit with the existence of a particular type of cell in the tissue used for inoculation have been unsuccessful.

Subsequent work, however, has proved that in a number of instances it has been possible to associate closely a positive result with the presence of the reticulum cell in lymphadenoma. This conclusion has been reached after systematic microscopical examination of Hodgkin lymph nodes that varied in their encephalitogenic activity towards the rabbit and by observing the incidence of various cell elements contained within them.

Attention was therefore focussed upon those tissues which showed the greatest cellularity in their composition. All sections were thus divided into two classes according to whether they had been derived from positive or strongly positive encephalitogenic material. An endeavour was then made to detect any significant histological differences between individual specimens, with the object of discovering if any particular cells or group of cells were to be linked with the reaction in the rabbit. Sections were stained by hæmatoxylin eosin and eosin methylene-blue as well as by Foot's method when available. The results indicated that gland tissue which contained numerous Hodgkin giant cells was found to be no more active than those which did not.

Likewise the relative incidence of neutrophilic and eosinophilic polymorphonuclear leucocytes did not appear to affect the results. The number of lymphocytes was also found to be unconnected with the reaction, and this was proved not only in certain lymphadenomatous glands in which numerous lymphocytes were noticed but also from the negative results recorded in lymphosarcoma, lymphatic and a-leukæmic leukæmia respectively.

On the other hand, it was repeatedly observed that large numbers of reticulum cells were encountered in those glands which gave a positive reaction. For example, in five specimens in which positive results were recorded it was observed that the tissue was composed principally of numerous large reticulo-endothelial cells with only occasional eosinophil and polymorphonuclear leucocytes intermingled among them.

The latter cells when stained by hæmatoxylin and eosin appear as large oval or fusiform structures, with a pale blue cytoplasm and a vesiculated nucleus, surrounded by a definite nuclear membrane. For a detailed description of their morphological characters the reader is referred to the careful description devoted to them by Pullinger (1932).

Whilst it is only reasonable to conclude that these cells (in an analogous manner to normal leucocytes) contained the encephalitogenic agent to the rabbit, it should be emphasised that in certain cases, although they were present in sections, the tissue failed to produce a strongly positive reaction in the rabbit. It would thus appear that the encephalitogenic agent is not always demonstrable in the reticulo-endothelial cells found in lymphadenoma, and its inconstancy in this tissue is similar to its occurrence or absence in various specimens of pus or leucocytes, *see* Gordon (1934) and MacKenzie and van Rooyen (1935).

It is of particular interest, however, that both the normal polymorphonuclear leucocytes as well as the reticulum cells observed in lymphadenoma should be found to contain a similar encephalitogenic agent towards the rabbit. It remains to be seen whether this finding would help to strengthen the views of Kidd and Turnbull (1908) and MacNalty (1928) and Pullinger (1932), who have postulated the local origin of the granular leucocytes in lymphadenoma, in contradistinction to the general opinion that they are the result of colonisation and deposition *via* the blood stream. Whichever of these possibilities ultimately proves to be correct, the current work

indicates the presence of a common characteristic existing between reticulum cell and the polymorphonuclear leucocyte. Pullinger (1932) quotes a case in which direct heteroplastic transformation of reticulum cells into myelocytes and promyelocytes occurred without passage through a common blood cell stage. This author also states earlier that " No further discussion on the exact nature of these changing cells can be pursued on account of lack of data."

Under the circumstances, the encephalitogenic property manifested by leucocytes, bone-marrow and the reticulum cells in Hodgkin's disease would appear to supply some information regarding the hitherto suspected relationship existing between these groups of cells.

In a paper dealing with the classification of diseases of reticulo-endothelial system, Ross (1933) advocated that a distinction should be established between hyperplasia affecting the undifferentiated or basic (true reticulum) of lymphatic glands, and the differentiated reticulo-*endothelial* cells (littoral cells) lining the sinus spaces. The same worker has placed lymphadenoma in the former category and states that the condition is a true reticulosis due to hyperplasia of undifferentiated cells possessing unrestricted potency for differentiation, *i.e.* follicular reticulum of lymph glands, Malpighian bodies of spleen and undifferentiated reticulum cells of connective tissue. Medlar (1931) has suggested that Hodgkin's disease and myelogenous leukæmia are genetically allied, and Pullinger has likewise concluded that the disease may be described as an extramedullary fibro-myeloid reticulosis.

There would appear to be no reason for the onset of these changes, and the failure to find a specific micro-organism strengthens the belief that the disease is of neoplastic origin. It must be remembered, nevertheless, that minute particles resembling elementary bodies found in certain virus diseases have also been seen in lymphadenomatous tissue by Gordon (1936). The latter has employed emulsions of sensitised elementary bodies for the treatment of the disease, and Warner (1936) has recently reported beneficial results following their administration in early cases. Warner's careful clinical observations regarding the effects of these vaccines are of great interest and for further details his publication should be consulted. Additional information, however, is necessary before it can be finally concluded that Hodgkin's disease is

a virus infection. Meanwhile, after due consideration of the findings of earlier investigators, and from the results of continuous research work into the ætiology of lymphadenoma extending over a period of years, the author has concluded his studies on this subject.

At the present time there is the strongest possible evidence to support the opinion that lymphadenoma is essentially a neoplastic process affecting lymphatic gland tissues.

REFERENCES.

Andrewes, F. (1902), *Trans. Path. Soc.*, London, liii., 305.
Barron, M. (1926), *Journ. Amer. Med. Assoc.*, lxxxvii., 1445.
Bartolozzi, M. (1934), *Bull. della Societa Med.-Chirurgica di Traviso*, i., 299.
Calvert, E. G. B., and Sanguinetti, H. H. (1933), *Brit. Med. Journ.*, i., 52.
Coles, A. C. (1934), *See* Gordon (1934).
Cunningham, W. F. (1915), *Amer. Journ. Med. Sc.*, lx., 868.
Davidson, L. S. P. (1934), *Med. Annual*, London, p. 230.
Davis, W. B. (1935), *Brit. Med. Journ.*, i., 423.
Friedemann, U. (1934), *Brit. Med. Journ.*, i., 517.
Gordon, M. H. (1932), *Rose Research on Lymphadenoma*, London.
Gordon, M. H. (1936), *Lancet*, ii., 65.
Gordon, M. H. (1934), *Proc. Roy. Soc. Med.*, xxvii., 49.
Greenfield, W. S. (1878), *Trans. Path. Soc.*, London, xxix., 272.
Kidd, F. A., and Turnbull, H. M. (1908), *Arch. Path. Inst. London Hospital*, ii., 404.
MacKenzie, I., and van Rooyen, C. E. (1935), *Brit. Med. Journ.*, i., 406.
Medlar, E. M. (1931), *Amer. Journ. Path.*, vii., 499.
MacNalty, A. S. (1928), *Rep. on Public Health and Med. Subjects*, London, Ministry of Health, No. 50.
Ogilvie, R. F., and van Rooyen, C. E. (1933), *Lancet*, ii., 641.
Ogilvie, R. F., and van Rooyen, C. E. (1934), *Journ. Amer. Med. Assoc.*, cii., 1842.
Paterni, L., Maroncelli, P., and Corsi, H. (1935), *Il Policlinico*, xli.
Pullinger, B. D. (1932), *Rose Research on Lymphadenoma*, London, p. 133.
Ritchie, H. (1935), *Med. Journ. Australia*, i., 197.
Rosenberg, D. H., and Bloch, L. (1936), *Journ. Amer. Med. Assoc.*, cvi., 1156.
Ross, J. M. (1933), *Journ. Path. Bact.*, xxxvii., 311.
Smith, E. C. (1935), *Lancet*, ii., 874.
Uhlenhuth, G. P., and Wurm, K. (1936), *Klin. Wschr.*, ii., 1025.
van der Hoeden, J., and Hulst, L. A. (1934), *Nederl. Tijdschr. v. Geneesk.*, lxxviii., 4305.
van Rooyen, C. E. (1933), *Brit. Med. Journ.*, i., 50.
van Rooyen, C. E. (1933), *Brit. Med. Journ.*, i., 664.
van Rooyen, C. E. (1933), *Brit. Med. Journ.*, ii., 562.
van Rooyen, C. E. (1934), *Brit. Med. Journ.*, i., 519.
Wallhauser, A. (1933), *Arch. Path.*, xvi., 522.
Warner, E. C. (1936), *Lancet*, ii., 417.

PRINTED IN GREAT BRITAIN BY OLIVER AND BOYD LTD., EDINBURGH

A SURVEY OF VIRUS INFECTIONS.

By C. E. van ROOYEN, M.D. (Edin.),

AND

A. J. RHODES, M.B., Ch.B., M.R.C.P. (Ed.).

(From the Department of Bacteriology, Edinburgh University.)

Reprinted from the "Glasgow Medical Journal," Jan., 1938, vol. cxxix, p. 1.]

A SURVEY OF VIRUS INFECTIONS.*

By C. E. van ROOYEN, M.D. (Edin.),

AND

A. J. RHODES, M.B., Ch.B., M.R.C.P. (Ed.).

(From the Department of Bacteriology, Edinburgh University.)

Introduction.

Recent work on virus infections of man and lower animals such as variola, vaccinia, psittacosis, herpes, ectromelia and fowl-pox, has shown that the specific ætiological agents responsible for these diseases appear as particulate and visible objects. These are now known as elementary bodies and are of historical interest to us since the elementary bodies of vaccinia and variola were first discovered in Scotland. The literary researches of Dr. Mervyn Gordon (1937) have revealed that they were first noticed by Dr. John Buist (1887) who practiced as public vaccinator in Edinburgh from 1877 to 1915. Professor Mackie informs us that the discovery was made by Buist when working in Professor Chiene's bacteriological laboratory which was not only the forerunner of its kind in the University but also

* The Substance of an Address and Demonstration delivered to the Glasgow Northern Medical Society on 9th December, 1937.

probably the first to be instituted in Great Britain. Unfortunately, Buist failed to recognize the ætiological significance of the minute granules he observed and called them the "spores of micrococci"—thus clearly appreciating that they were smaller than staphylococci (see Mackie and van Rooyen (1937).

Later these were observed by other workers, such as Calmette and Guerin (1901), Prowazek (1905) and Chaveau, the last of whom proposed the name of "elementary granules" for them, (see Gordon 1937).

In 1906 Professor Paschen of Hamburg published his investigations into the elementary bodies of vaccinia and variola. His subsequent studies conclusively proved the ætiological rôle of these granules beyond all shadow of doubt, as a result of which they have now been accorded world-wide recognition and universally named the Paschen bodies of vaccinia.

Following the work of Paschen on the elementary bodies of vaccinia, similar particles have been described in association with other virus diseases by later workers.

The Size of Viruses.

These granules vary considerably in their size and by means of filtration through collodion filters of graded porosity Elford (1931) and his co-workers have succeeded in measuring them. Thus—vaccinia virus has been found to be 125 to 175 $\mu\mu$ in dimension, herpes 100 to 150 $\mu\mu$, psittacosis 250 $\mu\mu$, and lymphogranuloma inguinale 150 $\mu\mu$ respectively.

The ability or otherwise of a virus particle to pass a particular filter depends on a number of factors, the principal of these being the average pore size of the membrane employed. By this means it is possible not only to gauge the probable size of virus bodies which are large enough to be visible by the ordinary microscope (x 1200) and measured by photomicrography but also those which are too small.

According to Coles (1929) the limit of visibility has been calculated to be 0·0740 μ to white light and 0·0673 μ to green light. The size of many virus particles estimated according to Elford's filtration methods, should therefore theoretically lie within the resolving powers of the ordinary microscope, provided suitable methods of staining could only be found to make them demonstrable.

Current work has tended to support this belief, for the elementary bodies of variola, vaccinia, psittacosis, herpes and molluscum contagiosum virus are clearly visable structures. There is no doubt that with improvements in technique and additions to our knowledge concerning the fundamental biological nature of viruses in general, the number of these agents visible by the ordinary microscope should tend to increase.

At the same moment it should be appreciated that there also exist many virus particles below 0·067 μ in size which are so small that they may never be seen by the ordinary microscope. The ultra-microscope perfected by Barnard (1936), employing a monochromatic source of ultra-violet light and an optical system constructed of quartz glass, promises to furnish a method whereby particles beyond the magnifying powers of the ordinary microscope may be photographed and measured. The figures obtained by Barnard closely correspond in size to those obtained by Elford's ultra-filtration. The high magnification and excellent definition provided by the ultra-violet light microscope also make it possible to elucidate finer morphological details in the structure that cannot be detected with lower magnifications.

The work of Ledingham (1932) has proved that elementary bodies can also be deposited from suspensions of infective material by means of high-speed centifrugation. Schlesinger (1934), Elford (1936), and McIntosh and Selbie (1937) and others have used centrifugation as a means for determining the size of elementary bodies. Thus, by subjecting a suspension of virus containing material to intense centrifugal force under conditions in which Stoke's Law is applicable, they have been able to calculate the probable particle size of the virus from its sedimentation rate.

Recently Hagemann (1937) has shown that if film preparations of virus bodies are treated with primulin (yellow 2 GS) or fluorochrome and exposed to ultra-violet light they tend to exhibit fluorescence. Himmelweit (1937) has used this principle for investigating the growth of vaccinia virus in the chorioallantoic membrane of the developing chicken embryo. The principle has many interesting possibilities in its application to virus research and these have yet to be explored.

OTHER BIOLOGICAL CHARACTERS OF ELEMENTARY BODIES.

Elementary bodies are thus not only definitely particulate but also display a variety of biological characteristics which suggest their relationship to the larger micro-organisms. Nauck and Paschen (1933) have demonstrated the growth and multiplication of vaccinia virus in tissue cultures and likewise Bland and Canti (1935) have made similar observations on psittacosis virus. As mentioned earlier, elementary bodies can be deposited, after centrifuging suspensions containing them at high-speed. Thus Ledingham (1932) and Amies (1934) have proved that the elementary bodies of vaccinia, fowl-pox, herpes zoster and varicella can be separated from infective tissue by centrifuging at 12,000 r.p.m. for about 35 minutes. The deposited bodies have then been washed by repeated centrifugation and obtained in a relatively pure state free from tissue protein material. These workers further showed that the elementary bodies of vaccinia, varicella and zoster may each be specifically agglutinated by the sera of patients recovering from these diseases, thus successfully demonstrating the presence of specific agglutinating antibodies in the sera of convalescent subjects. Specific agglutinin absorption tests analogous to those found in bacterial infections have also been used by Wilson Smith (1930) in vaccinia and herpes.

Gordon (1925), Craigie and Tulloch (1931) have used this information as the basis of an agglutination test for the diagnosis of variola. Craigie (1932) later showed that filtrates of vaccinial pulp contained a flocculable precipitable substance. Craigie and Wishart (1934, 1936) further demonstrated the dual antigenic structure of vaccinia virus and proved that it consisted of two fractions, one of which was thermolabile and the other thermostable. Specific complement-fixation tests were also described in vaccinia by Craigie and Wishart (1934). Likewise complement fixation reactions have been described in psittacosis by Bedson (1933), herpes by Bedson and Bland (1929), and in influenza virus by Fairbrother and Hoyle (1937).

A few viruses such as those of vaccinia, herpes and infectious myxomatosis of rabbits have been subjected to electro-cataphoresis and found to be negatively charged; this is another similarity to bacteria.

There is, therefore, little doubt that the elementary bodies of virus diseases represent the actual infective agent of the respective conditions in which they occur and are, moreover, forms of living matter. It seems incredible to believe that a form of life so small as the poliomyelitis virus which is 10 $\mu\mu$ in diameter could exist, but there appears to be no reason as to why it should be placed in a category separate to a larger visible virus (such as vaccinia) merely because of this feature. Modern work has tended to show that the so-called filterable and ultra-microscopic viruses represent a range of living agents that only differ from the bacteria by virtue of their minute size and their inability to multiply in cell-free media. Recently Laidlaw and Elford (1936) described a virus which they isolated from sewage and successfully cultivated on artificial media, an observation of great interest, since it had hitherto been generally regarded that living and surviving cells were essential for the growth and multiplication of viruses. The viruses admittedly possess certain distinctive characteristics such as the presence of inclusion bodies in invaded cells and their inability to multiply on artificial media, but apart from these features, in general, the trend of modern work tends to bring them into closer alignment with the visible bacteria.

It is not possible to leave this subject without making a brief reference to the work of Stanley (1936, *a* and *b*) on virus diseases of plants. He and his co-workers have isolated from plants affected by tobacco mosaic a crystaline protein which was capable of reproducing the disease when inoculated into other plants. The same substance, called heavy protein, has also been isolated from other plant diseases such as cucumber mosaic and potato X disease. More recently similar substances have been claimed to be recovered from yellow fever, Shope's infectious papillomatosis of rabbits, equine encephalomyelitis of horses and bacteriophage. The living nature of viruses has thus been challenged. The subject is a fascinating one and bristles with academic problems, and many interpretations have been advanced to explain these findings. One of them has been the view that the proteins isolated were protein enzymes which synthesized themselves after gaining access to the host's tissues. This work may yet profoundly influence our conceptions of viruses as

living entities and further researches are awaited with the keenest of interest.

Inclusion Bodies.

The occurrence of these bodies in infected tissues has proved to be one of the most characteristic features of certain virus infections. For convenience these bodies may be briefly classified as follows:—

A.—*Intracytoplasmic:*—

(1) *Eosinophilic.*—Guarnieri and intrafibroblastic bodies of vaccinia; Henderson-Patterson bodies of molluscum; Bollinger bodies of fowl-pox; Negri bodies of rabies; Marchal bodies of ectromelia; Prowazek-Halberstadter bodies of trachoma; Rivers' bodies of rabbit myxomatosis.

(2) *Basophilic.*—Psittacosis bodies (Bedson); Gamna-Favre bodies of L.G.I.; Japanese micro-corpuscles of L.G.I.; Thygeson bodies of inclusion conjunctivitis; Da Fano bodies of herpes and encephalitis.

B.—*Intranuclear, usually eosinophilic:*—

Lipschütz bodies of herpes; Torres' bodies of yellow fever; Rift valley fever bodies.

These bodies may be studied in a large number of ways:—

(1) Microchemical and staining tests may be carried out. The bodies may be tested, by their solubility in HCl, by the Feulgen technique for thymo-nucleic acid, and by the Macallum test for masked iron to exclude their nuclear origin; by intra-vitam Janus green, and by other methods to exclude mitochondria; and by the oxidase test to exclude their leucocytic origin. Certain inclusions show an affinity for brilliant cresyl-blue when this is added to preparations of tissue cells containing them. By these means it should usually be possible to decide whether the body is of nuclear, mitochondrial, leucocytic or other origin, but as regards whether it represents the virus itself further tests are necessary.

(2) Much help can be obtained from the study of virus-infected tissue cultures. The actively growing cells may show characteristic inclusion bodies, e.*g.*, the Guarnieri bodies of vaccinia (Haagen, 1931), the formation of which may be observed continuously either photographically or by cinematograph films, and the life cycle of psittacosis virus was worked out in this

way (Bedson and Canti, 1935). The formation of inclusion bodies may also be studied in infected eggs.

(3) Micro-incineration (Rector and Rector, 1933) is a method whereby thin films of tissue containing inclusion bodies are exposed to intense heat and ashed. Estimation of inorganic and organic material is then carried out, and it has been found that cytoplasmic inclusions contain abundant inorganic matter whereas intranuclear inclusions do not.

(4) Ultracentrifugation consists in centrifuging tissue containing inclusions at very high speed. In the case of herpetic intranuclear inclusions, for instance, it has been shown that they are lighter than chromatin and appear to exert an antagonistic effect upon it (Lucas and Herrmann, 1935).

(5) Micromanipulation is the supreme test to which every inclusion should be subjected. This has been performed in the case of fowl-pox, ectromelia and herpes, in which the disease has been reproduced with a single inclusion body (Woodruff and Goodpasture, 1929, 1930; Baumgartner, 1935). This demonstrates in a most convincing way that these bodies contain the living virus.

It appears that the great majority of inclusions represent aggregations of virus particles, often perhaps surrounded by a uniformly-staining coat formed from the enclosing cell or from the particles themselves. Certain inclusions such as the Gamna-Favre bodies of L.G.I. are, on the other hand, quite obviously of a different order, being probably nuclear or nucleolar extrusions.

Cultivation of Viruses.

Certain viruses will grow readily in the presence of living cells provided that they are suitably supported, nourished and warmed. For mere survival of the virus tissue filtrates will probably serve (Muckenfuss and Rivers, 1930) but for actual propagation intimate contact with whole and living cells is essential (Parker and Nye, 1925). The usual cells used in this work are obtained from rabbit testicle or cornea or from chick embryo tissues. The nutrient fluid varies, Tyrode's solution rabbit's serum, rabbit's plasma, and chick plasma all being used. If the fluid is to clot, then plasma must be employed. Spleen or embryo extract is usually used to promote growth, and clotting of plasma. As regards support, cells may be grown

on the under-side of coverslips, surrounded by nutrient fluid, as hanging drop preparations over hollow ground slides (the slide technique). Sometimes the tissue and nutrient fluid is used in larger amounts and enclosed within small glass or Carrel flasks (Maitland technique, Maitland *et al*, 1928, 1930, 1932). Generally speaking, virus is added to such cultures for one of two reasons, either to study the development of inclusion bodies or else for its propagation, and these purposes have been achieved in an ever-increasing number of viruses.

The developing chick embryo is also widely used in virus work (Woodruff and Goodpasture, 1931 ; Burnet, 1936).

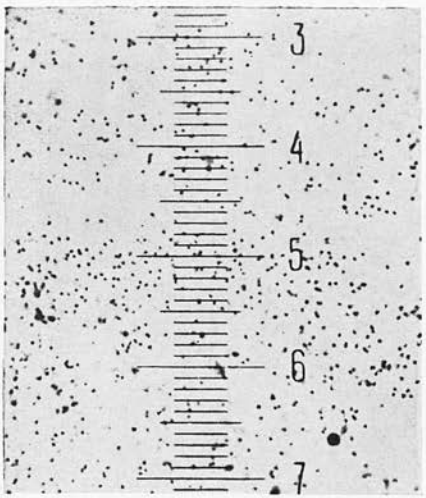
A small quantity of virus-containing material is injected through the shell of a fertile egg which has been incubated for 8 to 10 days. This material falls on to the chorio-allantoic membrane and there the virus proceeds to grow and produce a reaction characterized by the formation of numbers of pocks or plaques. After a few days' growth the egg may be opened and the membrane removed to constitute a rich yield of virus. A considerable number of viruses have been propagated in this fashion and a number of practical applications have been introduced. For instance, it is now possible to produce vaccinial material for human vaccination from the egg which is absolutely sterile and has the same biological properties as lymph produced from the calf (Stevenson and Butler, 1933, 1935).

Immunity to Virus Infections.

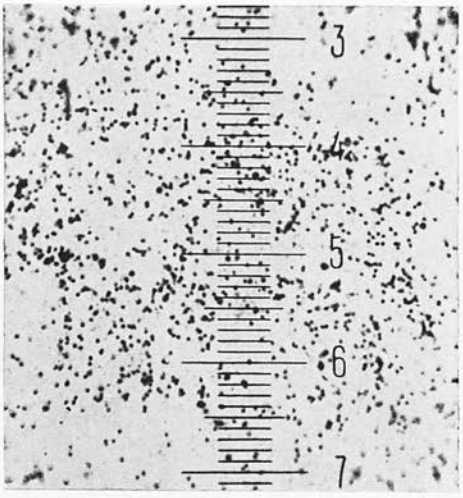
In certain instances, the serum of an individual who has passed through an attack of a virus disease will show specific neutralizing and other antibodies during convalescence. The subject would also be protected from further invasion, and at one time it was considered that resistance to a second attack was an essential criterion of this group of infections, but to-day no such generalization is possible.

It is still correct to say, however, that the immunity developed following smallpox, chicken-pox, measles, mumps, or poliomyelitis is of a high order and probably lasting in duration, but in a number of other diseases it is only of a transient nature.

For example, in herpes simplex, epidemic influenza and the common-cold the victim is frequently susceptible to recurrent attacks.



A—Photomicrograph made from a film preparation of vaccinia virus cultivated in the chorio-allantoic membrane of the chick embryo, showing Paschen or elementary bodies.



B—Similar picture made from a film of conjunctival secretion obtained from a rabbit infected with myxoma virus, showing elementary bodies.

C—Film showing a mixed culture of Staphylococci and *B. coli*. for comparison with the elementary bodies shown above.

All three have been stained by the same method, namely, Paschen's stain for elementary bodies. Each division on the micrometer scale = 1·2 μ. The total magnification = × 1222.

The therapeutic action of measles and poliomyelitis convalescent sera is too well-known to merit further description here, and whereas in these it is of value when given early on, it appears to be relatively useless when administered during the late stages of the illness.

The practical problem of storing human convalescent serum thus arises and this has yet to be satisfactorily dealt with. One of the best methods we have seen of preserving sera for long periods of time has been that used by Craigie and Tulloch by desiccating serum *in vacuo* by drying it from the frozen state. This principle of storing sera by desiccation has been placed on a commercial basis in America.

The mechanism of antivival immunity has been a topic of keen controversy and many different opinions have been expressed.

Andrewes (1930) showed that once a virus had gained access to a cell the addition of anti-serum failed to check its growth, whereas if the anti-serum were added before the virus then neutralization occurred.

Levaditi (1926) maintained that the immunity to herpetic infection is purely resident in the tissues, and Sabin (1935) has gone so far as to state that the action of an anti-serum is upon the cell and not the virus. The relative importance of humoral and cellular immunity thus appears to vary in different diseases. In all probability both mechanisms are involved.

The Modes of Transmission, Incubation Period, Localization and Excretion of Viruses.

Direct skin or mucus membrane contact is a common route of transmission, examples of which are seen in the case of variola, vaccinia, herpes, molluscum contagiosum, warts and lymphogranuloma inguinale. In others, droplet infection may cause spread, and this occurs in measles, mumps, influenza, psittacosis and poliomyelitis. Dried infective material deposited on domestic articles may also serve as an indirect means of transmitting many of these diseases.

The rôle of animal, insect and arthropod vectors is of great importance for many virus diseases of man are communicated *via* these agents. To quote a few cases—*A. aegypti* is respon-

sible for the transmission of yellow fever, *P. papatasii* for that of phlebotomous fever, and *A. aegypti* has also been accused of having transmitted herpes virus. Among animal virus diseases the number of them transmitted by insects and arthropods is very considerable. In the case of the blood-sucking creatures mentioned, these may either transmit the infection mechanically or else permit some cyclo-propagative phase to take place within their body.

A number of naturally occurring virus diseases of animals have been shown to be communicable to man. For example, in addition to rabies, cases of Rift Valley fever, louping-ill, and foot-and-mouth disease have lately been reported in man.

Another of the characteristic features met with in virus diseases is that of the definite incubation period which elapses, between the date of entrance of the virus into the host, and the period at which the signs and symptoms of illness develop. Great extremes are observed in the duration of this interval. For example in Rift Valley fever it may be as short as forty-eight hours, whereas in the case of warts and molluscum contagiosum the period may be months long. We ourselves have seen a case of rabies occurring in Scotland in a veterinary surgeon who had been licked by a rabid dog, before the victim left Africa, about six months previously.

This aspect of the battle against the spread of virus diseases is of growing importance and particularly in respect of the spread of yellow fever by air travel routes, a matter which has already received the serious and energetic attentions of the authorities concerned.

Having gained access to the tissues of its host, a virus may either cause a generalized invasion throughout the body or else exhibit a selective affinity for one of its tissues. Certain viruses such as those of variola-vaccinia, herpes, Rift Valley fever and yellow fever are pantropic in character and attack all three embryonic layers. Others, such as rabies and poliomyelitis virus which are neurotropic in character, and warts and molluscum contagiosum which are dermotropic in nature, tend to affect the ectodermal layer. Lastly, certain virus diseases of animals tend to be mesodermotropic in their distribution—for example, fowl-leukæmia, sarcoma and Shope's papilloma virus of the rabbit.

The excretion of virus is of interest to the Public Health worker as many of them may remain for considerable periods of time in tissues and individuals harbouring them are liable to act as carriers. The portals by which they leave the body tend to vary with individual infections; for example, variola, varicella and warts are shed from the skin; rabies, mumps, measles, the common cold, poliomyelitis, and influenza virus are found in the nasal or respiratory secretions. Yellow fever and dengue virus are present in blood; lymphocytic chorio-meningitis virus is excreted in the urine (Findlay, 1936); bacteriophage in the fæces.

Thus, medical measures designed to combat the spread of this group of diseases should be directed towards the early diagnosis and isolation of cases, and during the period of epidemics attention should be paid to maintaining the cleanliness of the skin and preservation of oral hygiene.

Active Immunization.

Human beings or animals can be made resistant to infection against certain viruses by immunizing them with either living attenuated virus or else dead virus suspensions. A multiplicity of different practices has been introduced to attenuate or otherwise modify the pathogenic effect of viruses, the two best known of these being the procedures adopted for the manufacture of rabies vaccine, and calf-lymph for human anti-smallpox vaccination. In connexion with the latter it is noteworthy that cultures of vaccinia virus cultivated in the chorio-allantoic membrane of the developing chicken embryo have already been used for Jennerian vaccination. The prophylatic efficacy of vaccine virus prepared by this method has yet to be proved, but the method is a valuable one whereby pure cultures of vaccinia virus may be obtained free from cocci and other organisms, and constitutes an important advance in this field.

During the last year the Hampstead workers (see Andrewes, 1937) made an effort to actively immunize human subjects against epidemic influenza by inoculating them with formolinized killed virus prepared from infected mouse lung. Although their experiments were not crowned with success, a number of important facts emerged from the results of their inquiries. For instance, they clearly showed that the inoculation of formo-

linised virus was followed by a rise in the antibody titre of the person's serum.

In America, Francis and Magill (1937) have used live influenza virus for their immunization of human subjects and reported good results. The therapeutic efficacy of vaccines such as these depend largely on the strain of virus employed. Andrewes (1937) recently drew attention to the fact that strains of virus isolated from various epidemics tended to be antigenically different in their composition, so that until more is known about the antigenic structure of influenza virus the preparation of influenza vaccines cannot be placed on a satisfactory basis.

Immunization against yellow fever supplies another example of what has comparatively recently been achieved in the control of virus infections. The method consists of injecting individuals with immune human yellow fever serum, followed later by a small dose of live neurotropic virus which has been maintained by repeated mouse brain passage (Sawyer, Kitchen & Lloyd, 1932). Findlay and MacCallum (1937) state that about 2,200 persons have thus been inoculated against yellow fever and only 48 of these have shown signs of hepatitis and jaundice at an interval of two to seven months after inoculation.

Immunization against virus diseases has also been performed by introducing live virus material parenterally *via* a route by which it is non-pathogenic. This has been done in the case of psittacosis virus by Rivers and Schwentker (1934, *b*), who have inoculated it subcutaneously into human subjects.

The value of chemotherapeutic agents such as prontosil has yet to be investigated in virus infections.

Diseases of the Skin.

Warts.—Clinicians differentiate a number of types of warts more on account of their site and differing appearance than for any difference in the ætiological agent which is the same for common, juvenile, plantar, genital, filiform and digitate warts. Laryngeal papillomata are also caused by the same virus (Ullmann, 1923; Ishikawa, 1936). The virus has not been demonstrated optically and can neither be cultured nor readily transmitted to animals. Warts often disappear spontaneously

but this does not appear to be due to an antibody effect (Brain, 1937).

Molluscum Contagiosum.—The ætiological agent of molluscum can be readily demonstrated in the form of elementary bodies in films from the characteristic lesions. The large eosinophilic inclusions were at one time thought to be hyalinized epithelial cells, but recent micromanipulation studies leave little room to doubt that they are actual accumulations of the elementary bodies themselves.

Herpes Febrilis.—All types of herpetic eruption (apart from Zoster) such as herpes febrilis, herpes labialis, herpes facialis, herpes cornealis and herpes genitalis are due to one and the same virus. Elementary bodies of small size, and characteristic large eosinophilic intranuclear inclusions have been described. The inclusions are probably composed of aggregates of the virus particles. The virus can be readily cultivated. It has a most characteristic action on animals; thus, if the cornea of a rabbit be infected an intense keratitis results with, in some cases, spread to the brain and death from encephalitis (Löwenstein, 1920; Doerr, 1920). The virus has been found in the saliva, conjunctival exudate and cerebro-spinal fluid of persons normal as well as herpetic (see Levaditi, 1926; Flexner and Amos, 1925). Approximately 75 per cent of normal human sera contain herpetic antibody (Andrewes and Carmichael, 1930), This antibody is said to be low in cases of recurrent herpes and absent in encephalitis lethargica (Gay and Holden, 1931). A recurrent herpes probably results in persons whose tissues are unusually susceptible to the virus, which is more or less continually carried in their saliva or elsewhere. Some success has attended the treatment of such cases with vaccines containing the virus and prepared from infected animals in the hope of raising the lowered antibody titre of the serum (Brain, 1936, 1937).

Herpes Zoster.—The ætiological agents have been demonstrated optically and occur as elementary bodies (Taniguchi, *et al*, 1934). These can be agglutinated by the serum of zoster as well as varicella convalescents (Amies, 1934). Complement fixation tests have also been carried out (Bedson and Bland, 1929). The virus cannot be cultivated and has not with certainty been transmitted to animals. The virus is probably quite unrelated to that of herpes febrilis but is closely connected if not identical with that of chicken-pox (varicella).

C. E. van Rooyen and A. J. Rhodes

Venereal Diseases.

(1) *Lymphogranuloma Inguinale* (L.G.I.). — This is the name in most general use to describe a disease previously known variously as climatic bubo, venereal bubo, malady of Durand and Favre, poradenitis and the sixth venereal disease. The disease is usually contracted in the Tropics or on the Continent. Cases are being reported however with increasing frequency where the infection has been contracted in Great Britain. Thus cases have occurred in Edinburgh, London and elsewhere (Stannus and Findlay, 1933; Earle, 1934; Whittaker, 1937; Batchelor, 1937). The disease is spread venereally and after an incubation period of from five days to a month a small, usually painless, primary ulcer or papule develops on the genitals. A few days later the regional lymph glands become enlarged and swollen, suppuration occurs and eventually the pus opens on to the surface of the groin. In females the affected regional glands frequently lie more deeply, in the pelvis, and this fact accounts for certain later disorders in which there is lymph stasis.

During this secondary stage general symptoms may be noted such as fever and malaise. Eosinophilia appears to be common (Coutts, 1936); also a mononucleosis may be found.

If untreated, the condition may heal spontaneously in the male, but this is relatively uncommon in the female, in which some months or years later tertiary lesions are found such as esthiomène, elephantiasis of the vulva, and rectal stricture. It is now becoming increasingly evident that the vast majority of cases of rectal stricture are due not to syphilis but to L.G.I.

The ætiological agent of the disease has been demonstrated optically by Miyagawa *et al* (1935), and other workers, in human and experimental lesions, and occurs in the form of small granules lying free or else inside epithelial and phagocytic cells. Inclusion bodies are known as Gamma-Favre bodies but represent merely nuclear material and not the virus itself.

The virus has been cultivated in Maitland's medium and by the egg (Tamura, 1934; Meyer and Anders, 1932; Miyagawa, 1935, *b.*). It can be readily transmitted to mice and monkeys with the production of a fatal meningo-encephalitis, to guinea-pigs with production of enlarged inguinal glands (Hellerström and Wassén, 1930; Levaditi, *et al*, 1932; Findlay, 1933-34).

Of invaluable aid in diagnosis is the Frei (1925) test, which consists in the intradermal injection of 0·2 cc. of pus from a known case of L.G.I., diluted 1/6 and heated to 60° C. for three hours. In positive cases an erythematous papule appears and by forty-eight hours is frequently 1·5 cm. in diameter. Central necrosis occurs, and this necrotic papule is still visible after seven days. The exact active principle in the Frei reaction is uncertain, but it cannot be the virus itself which is killed at 60° C. in a few minutes. Frei antigen has been used successfully in treatment, injected either intradermally or intravenously in repeated doses (Batchelor, 1937).

(2) *Herpes Genitalis.*—This disease is most common in cases of chronic gleet and prostatitis, and is due to the same agent as herpes febrilis which is described above.

(3) *Genital Warts* is the best term to use for all warty growths of the genital region, instead of condylomata acuminata, gonorrhœal warts and venereal warts. The lesions are due to the same filterable agent as causes ordinary skin warts (described above) and not to syphilis, gonorrhœa or other condition.

Respiratory Diseases.

Influenza.—Extensive work (recently reviewed by Andrewes, 1937) has been carried out during the past four years on a virus originally isolated from cases of epidemic influenza by Laidlaw and his associates at the National Institute for Medical Research, London. The influenza bacillus plays no essential part in the ætiology of this condition, which is a pure virus infection. A world-wide distribution of the virus has been shown as it has been found in countries as widely separated as the U.S.A., Australia, Germany, Russia and Holland. Up to now it has generally been accepted that these strains are all very closely related, but recently it has been suggested that there may, in fact, be a certain amount of antigenic variation between them (Magill and Francis, 1936; Andrewes, 1937). The virus has been measured and falls between 0·08 and 0·12 μ in diameter.

The virus can be readily grown in tissue culture and in the egg. It can be transmitted to ferrets by intranasal inoculation of filtered throat-washings of the human case with the production of a purulent rhinitis transmissible in series. If injected under an anæsthetic, pneumonia may occur. Mice can also be

infected, most readily from ferret-material, but also probably in certain cases direct from man, with the production of lung lesions. The virus after passage in ferrets has proved capable of causing influenza in human beings (Smith and Stuart-Harris, 1936; Smorodintseff *et al*, 1936).

Virus-neutralizing antibodies are absent from the serum of acute cases but appear during convalescence. Active immunity can be conferred on ferrets and mice with subcutaneous injections of living virus (purified by washing on collodion membranes) or with formolized virus (Andrewes and Smith, 1937).

Naturally these results have led up to the question of the vaccination of man. Injections of living virus, formolized virus and egg passage virus have all been used by various workers (*e.g.*, Andrewes, 1937; Francis and Magill, 1937), and certainly rises of antibody titre have been noted. As yet, however, there has been no convincing proof that influenza can be prevented, but further research, it is hoped, may achieve this result. Anti-viral serum has been produced from the horse, and this too may yet prove its value (Laidlaw *et al*, 1935).

Before concluding this problem a word must be said about swine influenza. This has been extensively studied, mainly in America, by Shope (see Shope, 1931, 1935), and is an influenzal disease of pigs in the ætiology of which both swine-influenza virus and *B. influenzæ-suis* are concerned. The virus acting alone, however, can be transmitted to ferrets and produces a picture identical to that due to the human virus. For this and other reasons a very close relationship between the two viruses has been claimed, and it has been suggested that the virus now known as the Shope swine virus was originally the human strain that caused the 1918 pandemic.

The view has been largely based on a study of the virus-neutralizing power of children's and adult's sera, since no neutralizing bodies to the swine virus were found in children although they were frequently present in adults, i.e., those who presumably acquired them in 1918 (Andrewes *et al*, 1935; Shope, 1936).

Common Colds.—This disease has not been so extensively studied as has influenza. There appears to be no doubt, however, that a filterable agent can be isolated from human cases, which can be grown in tissue culture and transmitted to

apes (see Dochez *et al*, 1936). Unfortunately, no smaller animals have been found susceptible (with the exception of the hedgehog) and this fact is largely responsible for the poor progress that has been made.

Mumps.

Filtrates of saliva from infected persons have been injected into the Stenson's duct of monkeys and produced a swelling of the parotid closely similar to human mumps. The most recent work (*e.g.*, Johnson and Goodpasture, 1935) has thus confirmed the view expressed by Gordon (1914) that mumps was due to a filterable virus.

Psittacosis.

The old view that this disease of parrots and man was due to a cultivable bacillus has been completely reversed by the researches of Bedson, Bland and their co-workers (see Bedson 1936; Bedson and Bland, 1934). Bedson has found that the ætiological agent undergoes a definite life cycle, and that this can be readily followed either in experimentally infected mice or in tissue cultures. The smallest form is coccal in appearance and about 0·25 to 0·3μ in diameter. These forms multiple inside endothelial cells and eventually form a fused mass or morula which eventually breaks down and liberates numbers of elementary bodies. All forms of the virus stain basophilic with Giemsa or Castaneda. Ultrafiltration shows the size of the bodies to measure 0·2 to 0·3μ. The virus can be grown in tissue cultures and in eggs.

The disease can only be diagnosed with certainty by bacteriological means (Levinthal and Bedson, 1937). During the first four days of illness defibrinated blood should be injected into a number of mice. If these do not die some should be killed after ten to fourteen days and smears from the spleen examined for virus bodies. The remaining animals are killed after four weeks.

Pleural exudate and sputum may yield the virus on injection, if necessary repeated, of mice.

Complement fixation occurs between convalescent serum and infected mouse spleen antigen (Bedson, 1933).

Infectious Mono-nucleosis (Glandular Fever).

The ætiological agent of this disease is unknown, but has

been claimed to be a virus. The most important recent advance is in diagnosis. The serum of cases agglutinates suspensions of sheep's red blood cells in high dilution (up to 1/1,000 due to a heterophil antibody different in nature to these antibodies found in normal serum and in cases of serum disease (Davidsohn, 1937).

Animal Diseases Rarely Transmissible to Man.

Louping-Ill.—This disease occurs commonly in sheep in Scotland and is characterized by symptoms of ataxia. The virus is spread by ticks, is from Ø15 to Ø20 μμ in diameter and can infect mice and monkeys experimentally. Of main interest however are a number of reports of the disease occurring in laboratory workers (Rivers and Schwentker, 1934, *a*). These patients showed symptoms of encephalitis but only very rarely ataxia. The virus was not isolated from the blood, and after recovery their sera neutralized louping-ill virus. Finally, it has been suggested (Perdrau, 1936) that a type of encephalitis occurring in Australia (X disease), possibly contracted from sheep, may be louping-ill.

Foot and Mouth Disease.—The virus measures 0·08 to 0·12 μ in diameter, can be grown in tissue culture, and infects guinea-pigs and other small animals experimentally. Cases of the disease have been reported in man, infectious material from animals entering through cuts and abrasions or perhaps in milk. After a short incubation period the oral mucosa becomes swollen and congested with numerous small vesicles. In severe cases similar eruptions are found on the hands or feet (Clough, 1915).

"*Orf.*"—This disease is commonly found in sheep as contagious pustular dermatitis. Cases have recently been reported in persons exposed to infection from such animals. The lesions often appear on the face as large rounded painless swellings. It has been suggested that this disease is due to a filterable virus (Peterkin, 1937).

Rift Valley Fever.—This disease attacks sheep, cattle and other animals in Kenya (Daubney and Hudson, 1936). The virus measures from Ø25 μμ to Ø35 μμ in diameter, has been cultivated in tissue culture, and infects mice and other animals experimentally. Characteristic intranuclear inclusions appear within a few hours in infected mice. Persons exposed to

infection in that part of the world, and laboratory workers, may develop a febrile disorder characterized by severe headache and body pains. Intranuclear inclusions may be demonstrated in the liver of fatal cases.

Diseases of the Central Nervous System.

Encephalitis Lethargica.—A number of workers have isolated a virus from cases of encephalitis lethargica which in behaviour is identical with the characteristic virus of herpes. Much experiment was directed to proving the identity of the two viruses, which is now generally admitted. Less attention was paid, however, to proving that the virus isolated from cases of encephalitis was actually the primary infecting agent and not just an accidental contaminant. It is known that herpes virus may be widely distributed in the bodies of herpetic as well as healthy persons. It is possible, therefore, that the cases from which the so-called encephalitis virus were isolated were merely carriers of the herpes virus. The solution is therefore in abeyance and the problem to be solved is—Can the herpes virus cause encephalitis in man?—for that it can do so in animals is a matter of common knowledge. Certain serological observations have been directed towards elucidating this question. Thus, while approximately 75 per cent of normal human sera neutralized the herpes virus in vitro (Andrewes and Carmichael, 1930) this property was not found in sera tested from cases of acute encephalitis (Gay and Holden, 1931). The precise interpretation of this latter finding is difficult, and particularly whether or not it implies that encephalitis occurs only in those who possess no immunity to herpes. For the present, therefore, the pathological rôle of herpes virus in encephalitis is not proven and the exact ætiology is uncertain.

St. Louis Encephalitis.—In marked contrast to the unsatisfactory state of affairs in encephalitis lethargica a specific virus has been isolated from an outbreak of encephalitis that occurred in St. Louis, U.S.A., in 1933. The virus measures 0·2 μ to 0·3 μ in diameter and infects monkeys and mice experimentally. The reactions produced in the monkey are closely similar to those found in the human cases.

Rabies.—No elementary bodies of rabies have been demonstrated and the virus has not with certainty been grown

in tissue culture. Street virus produces the characteristic Negri bodies in the cytoplasm of nerve cells. These bodies have not been studied by modern methods and it is uncertain whether they represent aggregates of the virus or degenerative material. It has been shown that rabies may be spread in certain parts of the world by bats, infected cattle possibly forming a reservoir-host of the virus (Hurst and Pawan, 1931).

The prevention of rabies by means of various vaccines is now firmly established. It should be remembered that with modern means of transport it is quite possible for cases of rabies to be infected abroad and to develop the disease in this country.

Poliomyelitis.—The virus of poliomyelitis is said to measure 8 to 10 $\mu\,\mu$ in diameter; it can be propagated in tissue culture and infects monkeys experimentally. The disease is spread mainly by carriers. The virus passes to the central nervous system *via* the olfactory axis cylinders, and in the case of monkeys injected intravenously the central nervous system is still infected by the same route.

Virus-neutralizing bodies have been found widely distributed in adult sera (Aycock and Kramer, 1930), and it is obvious that if this be due to active immunization then the virus must be very prevalent, without actually causing infection. As regards prevention of the disease, vaccines prepared from animals and containing viable virus have been shown to be extremely dangerous, as fatal results may follow their use. There is no very convincing evidence that killed vaccines are of value in preventing the disease. It appears, however, from monkey experiments, that picric acid and alum instilled into the nose is a most effective prophylactic against infection (Armstrong, 1936).

The position of serum therapy remains unaltered; that is to say, if the serum is to be of any value it must be injected in the preparalytic stage.

Benign Lymphocytic Chorio-Meningitis.—This disease is being increasingly recognized in this country (for a recent review see *B.M.J.*, 1936, *2*, 235). This virus has been grown in the egg and infects, monkeys, mice, and guinea-pigs experimentally. The virus may be found endemically in certain laboratory stocks of mice, but whether mice transmit the disease to man has not been determined. Clinically the disease presents features of an acute meningitis with a lymphocytosis in the cerebro-spinal

fluid (see Hughes, 1937), and may be confused with tuberculous meningitis, but film examination shows no organisms and culture proves sterile. Animal inoculations should be carried out with cerebro-spinal fluid of all such cases, and intracerebral injection of mice results in their death in about ten days with spastic lesions of the limbs. The virus may be found in the blood, in the early stages, and convalescent serum has virus-neutralizing properties.

The Acute Exanthemata.

There has been little fresh knowledge introduced in regard to this group of infections. The diagnosis of variola by Gordon, Craigie and Tulloch's flocculation test has already been alluded to, and it may be mentioned that small-pox may be diagnosed by means of Paul's test. This is performed by scarifying the cornea of a rabbit with variolous material and then keeping the animal under observation until keratitis develops, when the test is read as positive. The question of post-vaccinial encephalitis calls for no fresh comment apart from the fact that a somewhat similar state of affairs exists in the case of yellow-fever immunization with neurotropic virus, which has occasionally invaded the central nervous system in a few susceptible subjects.

It has been shown that certain toxic substances such as diphtheria and tentanus toxis do not pass the endothelial lining of the cerebral capillaries, whereas lamb-dysentery toxin and cobra venom do so. This selective mechanism is known as the blood-brain barrier (Friedmann and Elkeles, 1934) and may in certain circumstances break down, thus exposing the nervous system to microbic attack.

The relationship of varicella to zoster has long been the subject of clinical discussion and at the present time further proof in favour of the ætiological relationship of these diseases to one another has been advanced by Amies (1934). He has shown that the elementary bodies present in the vesicle of of one of these conditions may be specifically cross-agglutinated by sera obtained from convalescent cases from the other condition.

Reference has already been made to the prophylactic efficacy of convalescent measles antiserum when administered before the sixth day of the illness (Nobarro and Signy, 1931). The course of the disease may thus be modified by the early use of serum.

THE RHEUMATIC DISEASES.

Schlesinger, Signy and Amies (1935) have demonstrated the presence of elementary bodies in pericardial effusions from cases of acute rheumatic fever. They have also demonstrated the presence of specific agglutinins in the sera of convalescents and hence claim that rheumatic fever may be a virus disease. Eagles (1937) later showed that the sera of patients suffering from rheumatic fever, chorea and rheumatoid arthritis agglutinate virus suspensions from the corresponding diseases — thus suggesting a virus ætiology similar to that of rheumatic fever.

INCLUSION CONJUNCTIVITIS.

This is a condition of some interest which has been described in America by Thygeson (1934). It is supposed to be acquired by swimming in infected public baths, and hence the description, "swimming bath conjunctivitis." There is also some indication that the disease may be spread venereally and new-born children may develop it from an infected mother. The pathological features of the disease consist of conjunctivitis, in the secretion of which collections of small basophilic intracytoplasmic inclusion bodies are found in the epithelial cells. The ætiological agent may be a virus or else a member of the group of rickettsiæ. So far, cases have not been described in this country.

In concluding this survey of current developments in the field of virus diseases we fully realize that much has had to be omitted, but trust that we have said sufficient to arouse interest in this most fascinating branch of medical research.

REFERENCES.

Amies, C. R., 1934, *Brit. Journ. Exp. Path.*, *15*, 314.
Andrewes, C. H., 1930, *Journ. Path. Bact.*, *33*, 301.
Andrewes, C. H., 1937, *Brit. Med. Journ.*, *2*, 513.
Andrewes, C. H., and Carmichael, E. A., 1930, *Lancet*, *1*, 857.
Andrewes, C. H., Laidlaw, P. P., and Smith, W., 1935, *Brit. Journ. Exp. Path.*, *16*, 566.
Andrewes, C. H., and Smith, W., 1937., *Brit. Journ. Exp. Path.*, *18*, 43.
Armstrong, C., 1936, U.S. Public Health Report, *51*, 241.
Aycock, W. L., and Kramer, D., 1930, *Journ. Prev Med.* (Baltimore), *4*, 189, 201.
Barnard, J. E., 1936, *Journ. Roy. Micr. Soc.*, *56*, 365.
Batchelor, R. C. L., 1937, *Edin. Med. Journ.*, N.S. (4th), *44*, 129.
Baumgartner, G., 1935, *Schweiz. Med. Wchschr.*, *16*, 759.
Bedson, S. P., 1933, *Brit. Journ. Exp. Path.*, *14*, 162.
Bedson, S. P., 1936, *Brit. Journ. Exp. Path.*, *17*, 109.

Bedson, S. P., and Bland, J. O. W., 1929, *Brit. Journ. Exp. Path, 10,* 393.
Bedson, S. P., and Bland, J. O. W., 1934, *Brit. Journ. Exp. Path 15,* 243.
Bland, J. O. W., and Canti, R. G., 1935, *Journ. Path. Bact., 40,* 231.
Brain, R. T., 1936, *Brit. Journ. Derm. Syph., 48,* 21.
Brain, R. T., 1937, *Brit. Med. Journ., 2,* 1064.
Buist, J., "Vaccinia and Variola," London: Churchill, 1887.
Burnet, F. M., Med, Res. Counc. Spec. Rep., No. 220, London, 1936.
Calmette, A., and Guerin, C., 1901, *Ann. Inst. Past. 15,* 166.
Clough, P. W., 1915, *Bull. Johns Hopkin's Hospital, xxvi,* 351.
Coles, A. C., 1929, *Brit. Med. Journ., 11,* 91,
Coutts, W. E., 1936, *Journ. Trop. Med. and Hyg., 39,* 113.
Craigie, J., 1932, *Brit. Journ. Exp. Path., 13,* 259.
Craigie, J., and Tulloch, W. J., Med. Res. Coun. Spec. Rep., No. 156, London, 1931.
Craigie, J., and Wishart, F. O., 1934, *Trans. Roy. Soc. Can., S.5.,* 91.
Craigie, J., and Wishart, F. O., 1936, *Journ. Exper. Med., 64,* 803,
Daubney, R., and Hudson, J. R., 1936, *Journ. Compar. Path., 49,* 63.
Davidsohn, I., 1937, *Journ. Amer. Med. Assoc., 108,* 289.
Dochez, A. R., Mills, K. C., and Kneeland, Y., 1936. *Journ. Exper. Med., 63,* 559.
Doerr, R., 1920, *Klin. Monatsbl. f. Augenh., 65,* 104.
Earle, K. V., 1934, *Lancet, 2,* 137.
Eagles, G. H., 1937, *Lancet,* 2, 421.
Elford, W. J., 1931, *Journ. Path. Bact., 34,* 505.
Elford, W. J., 1936, *Brit. Journ. Exp. Path., 17,* 399.
Fairbrother, R. W., and Hoyle, L., 1937, *Journ. Path. Bact., 44,* 213.
Findlay, G. M., 1933-34, *Trans. Roy. Soc. Trop. Med. and Hyg., 27,* 35.
Findlay, G. M., 1936, *Proc. Roy. Soc. Med., 29,* 563.
Findlay, G. M., and MacCallum, F. O. (1937), *Trans. Roy. Soc. Trop. Med. and Hyg., 31,* 297.
Flexner, S., and Amoss, H. L., 1925, *Jour. Exper. Med., 41,* 215.
Francis, T., and Magill, T. P., 1937, *Jour. Exper. Med., 65,* 251.
Frei, W., 1925, *Klin. Wchnschr, 4,* 2148.
Friedmann, U., and Elkeles, A., 1934, *Lancet, 1,* 775.
Gay, F. P., and Holden, M., 1931, *Jour. Amer. Med. Assoc., 96,* 2028.
Gordon, M. H., Rep. Loc. Govt. Board, N. S., 96, 1914.
Gordon, M. H., Med. Res. Counc. Spec. Rep., No. 98, London, 1925.
Gordon, M. H., 1937, *Edin. Med. Journ.,* N. S. (4th), *44,* 65.
Haagen, E., 1931, *Zbl. f. Bakt., Abt. I. Orig., 120,* 304.
Hagemann, P. 1937, *Munch. Med, Wchnschr., 84,* 761.
Hellerström, and Wassén, E., VIII Congres International de Dermatologie et de Syphiligraphie, Copenhagen, 1930.
Himmelweit, F., 1937, *Lancet, 2,* 444.
Hughes, W., 1937, *Brit. Med. Journ., 1,* 1063.
Hurst, E. W., and Pawan, J. L., 1931, *Lancet, 2,* 622.
Ishikawa, K., 1936, *Fukuoka Acta. Med., 29,* 87.
Johnson, C. D., and Goodpasture, E. W., 1935, *Amer. Journ. Hyg., 21,* 46.
Laidlaw, P. P., Smith, W., Andrewes, C. H., and Dunkin, G. W., 1935, *Brit. Journ. Exp. Path., 16,* 275.
Laidlaw, P. P., and Elford, W. J., 1936, *Proc. Roy. Soc. B., 120,* 292,
Ledingham, J. C. G., 1932, *Journ. Path. Bact., 35,* 140.

A Survey of Virus Infections

Levaditi, C., "L'Herpès et le Zona," Masson: Paris, 1926.

Levaditi, C., Ravaut, P., and Schoen, R., 1932, *C. R. Soc. Biol., cix, 285.*

Levinthal, W., and Bedson, S. P., Rep. P. H. Med. Subj., London, 1937.

Löwenstein, A., 1920, *Klin. Monatsbl. f. Augenh., 64,* 315.

Lucas, A. M., and Herrmann, 1935, *Amer. Journ. Path., 11,* 969.

Mackie, T. J., and van Rooyen, C. E., 1937, *Edin. Med. Journ., 44,* 72.

Magill, T. P., and Francis, T., 1936., *Proc. Soc. Exp. Biol. and Med., 35,* 463.

Maitland, H. B., and Laing, A. W., 1930, *Brit. Journ. Exp. Path., 11,* 119.

Maitland H. B., and Maitland, M. C., 1928, *Lancet, 2,* 596.

Maitland, H. B., Laing, A. W., and Lyth, R., 1932, *Brit. Journ. Exper. Path., 13,* 90.

McIntosh, J., and Selbie, F. R., 1937, *Brit. Journ. Exper. Path., 18,* 162.

Meyer, K., and Anders, H. E., 1932, *Klin. Wchnschr.,* 318.

Miyagawa, Y., *et al,* 1935 (a), *Jap. Journ. Exper. Med., 13,* 1.

,, ,, (b) ,, ,, ,, 733.

Muckenfuss, R. S., and Rivers, T. M., 1930, *Journ. Exper. Med., 51,* 149.

Nabarro, D. N., and Signy, A. G., 1931, *Brit. Med. Journ., 2,* 599.

Nauck, E. G., and Paschen, E., 1933, *Zbl. f. Bakt. Abt. I. Orig., 128,* 171.

Parker, F., and Nye, R. N., 1925, *Amer. Journ. Path., I,* 325.

Paschen, E., 1906, *Munch. Med. Wchnschr., 53* (2), 2391.

Perdrau, J. R., 1936, *Journ. Path. Bact., 42,* 59.

Peterkin, G. A. G., 1937, *Brit. Journ. Derm. Syph., 49,* 492.

Prowazek, S., 1905, *Arb. aus. d. Kaiserol. Gesundheitrz., 22,* 535.

Rector, L. E., and Rector, E. J., 1933, *Amer. Journ. Path., 9,* 587.

Rivers, T. M., and Schwentker, F. F., 1934 (a), *Journ. Exper. Med.,* 59, 669.

,, ,, ,, ,, (b), ,, ,, *60,* 211.

Sabin, A. B., 1935, *Brit. Journ. Exper. Path., 16,* 70.

Sawyer, W. A., Kitchen, S. F., and Lloyd, W., 1932, *Journ. Exper. Med., 55,* 945.

Schlesinger, M., 1934, *Kolloidzschr., 67,* 135.

Schlesinger, B., Signy, A. G., and Amies, C. R., 1935, *Lancet, 1,* 1145.

Shope, R. E., 1931, *Journ. Exper. Med., 54,* 373; 1935, *62,* 561; 1936, *63,* 669.

Smith, W., 1930, *Journ. Path. Bact., 33,* 273.

Smith, W., and Stuart-Harris, C. H., 1936, *Lancet, 2,* 121.

Smorodintseff, A. A., *et al,* 1936, *Lancet, 2,* 1383.

Stanley, W. M., 1936 (a), Proc. 2nd Internat. Cong. Microbiol., Harrison, London.

,, ,, (b), *Phytopath., 26,* 305.

Stannus, H. S., and Findlay, G. M., 1933, *Lancet, 1,* 358.

Stevenson, W. D. H., and Butler, G. G., 1933, *Lancet, 2,* 228; 1935, *Bull. Off. Int. Hyg. Pub., 27,* 48.

Taniguchi, T., Hosokawa, M., Kuga, S., Masuda, Z., 1934, *Jap. Journ. Exper. Med., 12,* 101.

Tamura, J. T., 1934, *Journ. Amer. Med. Ass., 103* (1), 408.

Thygeson, P., 1934, *Amer. Journ. Ophth., 17,* 1019.

Ullmann, E. V., 1923, *Acta Oto-laryngol., 5,* 317.

Whittaker, N., 1937, *Lancet, 2,* 1183.

Woodruff, A. M., and Goodpasture, E. W., 1929, *Amer. Journ. Path., 5,* 1; 1930, *6,* 713; 1931, *7,* 209.

GLASGOW: PRINTED BY ALEX. MACDOUGALL