

STUDIES IN MICROBIC DISSOCIATION AND VARIATION

WITH SPECIAL REFERENCE TO THE

ACID-FAST AND THE DIPHTHEROID BACILLI

by

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I N T R O D U C T I O N

More than fifty years ago the belief was current among bacteriologists that microorganisms could change readily from one form to another and exhibit in this process a remarkable variability of size and shape. Frequently such pleomorphism was explained by the fact that cultures exhibiting it were contaminated by spores, and Cohn (1875) and Koch, in his various writings, put forward evidence to show that the bacterial form was fixed and stable. So convincing was this evidence that the Cohn-Koch doctrine of monomorphism has held sway in bacteriological thought until recent years. Undoubtedly such a view has proved of value, since, in establishing the fact that a specific microorganism was associated with a recognized clinical condition and could be isolated from persons suffering from that disease a firm basis was laid for the study of bacterial infections and for evolving, as a result of that study, suitable methods of prophylaxis and treatment. Such a belief naturally resulted in a systematic classification of bacterial types.

Recently, however, equally convincing evidence has been put forward in favour of the view that the majority/

majority, if not all, known bacterial species occur in at least two forms, one of which may be so different in its various characteristics from the so called "normal" or generally recognized type that, from the point of view of monomorphism, systematists would be justified in classifying it as a different species. Frequently cultures containing such forms have been discarded as contaminated, and had it not been for occasional reports by careful investigators "Microbic Dissociation", as the phenomenon is now called, would not yet be established as an important fundamental principle in bacteriology.

Briefly "Microbic Dissociation" as it is understood to-day may be regarded as the transformation of a "pure-line" culture into one or more subtypes, differing from the original culture in one or more of those bacterial forms or reactions which, classified broadly, come under the headings of morphology, cultural characteristics, physiological behaviour, antigenic structure and virulence.

Microbic dissociation may be of two types. The first includes those modifications or fluctuations of a purely temporary nature due mainly to variation of environmental/

environmental conditions such as temperature, moisture, hydrogen-ion concentration, or harmful substances incorporated in the medium. Subsequent transference to favourable conditions results in a return to the "normal". Such variations in character are not maintained in subsequent generations and therefore, so far as is known at present, are of only minor importance. The second type of dissociation is that by which fixed or stable variants are developed. These persist in the newly acquired form during subsequent subcultivation which may extend over considerable periods of time and which, therefore, involve millions of generations of bacteria. Frequently variants occurring under the given conditions of observation are not known to return to the original form and to these in early literature the term "mutant" was applied. It seems doubtful, however, whether there is justification for this nomenclature since in most instances only a limited number of stimuli have been applied in the attempt to induce reversion and the term "mutant" in its usual biological sense is not applicable to bacterial variation.

Nomenclature.

Reference to variation in one form or another has appeared/

appeared in literature from time to time for many years. A great advance was made in the study of the subject by Arkwright (1920; 1921) who suggested a system of nomenclature based on the appearance of colony types of the typhoid-dysentery groups of organisms and associated with these certain other characteristics, thus establishing a basis for subsequent investigation, and at the same time throwing some light on existing information. The predominant round, glistening colony he named "S" or "smooth" and the large, irregular variant "R" or "rough". Previous to this Weil and Felix (1917) described a spreading (hauch) and non-spreading (ohne hauch) form of B. proteus and applied to them the symbols "H" and "O" which are now generally used to designate two different types of antigen. DeKruif (1921a) working with the bacillus of rabbit septicaemia classified the main variants according to their type of growth in broth: "D" (diffuse) and "G" (granular) which may be regarded as synonymous with S and R.

"I" and "O" have been used to represent intermediate types but these are so numerous and unstable that symbols are not readily applicable. No doubt such forms have often been observed but only rarely have they/

they been reported in detail. The descriptions of variants given by Eisenberg (1905-18) for many different organisms are outstanding and suggest that by placing too great emphasis on certain types the significance of other forms may be overlooked.

At the present time, therefore, the two main dissociates of any bacterial species are usually referred to as S and R. Hadley (1927) pointed out that in certain instances the colonies of variants classified as S in virtue of various other characteristics such as antigenic structure, virulence and ready emulsibility in normal saline were of a rough and irregular structure, and he suggested that S and R should denote "sensitive" and "resistant" since there is some foundation for the belief that the S form is the more sensitive to environmental conditions. This suggestion, however, is definitely open to criticism as it seems hardly likely that variation is entirely dependent on external stimuli. When the mechanism of the phenomenon is more fully understood no doubt a more suitable nomenclature will be devised.

Variation in Colony Form.

Since S and R colonies of one species may differ widely from S and R colonies of another species even although/

although they are closely related, it is impossible to give a general description which could appropriately be applied to the two main variants of all microorganisms. The colonies of each species require individual portrayal. For convenience and brevity I shall classify variants into three groups: (1) the so-called "normal" or S colony; (2) the so-called R variant; (3) intermediate types.

In the class of Gram-negative intestinal bacilli the smooth, round, entire, convex and glistening appearance of the most prevalent type of colony resulted in the application to it of the term S or smooth, a nomenclature equally suited to various other organisms such as B. diphtheriae, the streptococci and the pneumococcus. It is not, however, in keeping with the characteristics of certain species of acid-fast organisms or spore-formers which tend to have a folded and complex structure although corresponding in many other respects to the S type of intestinal organisms.

Rough, matt or irregular colonies may occur spontaneously or be induced artificially in cultures composed usually of S organisms. These so-called R forms may differ from the original not only in the appearance of their colonies but also in many other characteristics/

characteristics. In the majority of bacterial species this variant is recognizable by its structure but confirmation should be made by additional tests to avoid confusion with intermediate types. In some instances the R is the "normal" or predominant variant as in the case of B. anthracis and, according to Todd (1928), in the strains of streptococci with which he worked. Exceptions of this sort suggest still more strongly that classification according to the terms S and R in which S usually connotes the commonly occurring form of an organism is unsatisfactory.

Into the third group may be placed all those types in every stage of gradation between the two extremes S and R. The trend toward the extreme may be expressed by the addition of figures, as for instance, R_1 , R_2 , R_3 . Part of the confusion existing in the literature is possibly due to some of these intermediate forms having been regarded as extreme R variants (probably because of their colony structure) without sufficient justification for the application of the term. The intermediate forms vary greatly in their degree of stability and for this reason have not received the study necessary for an understanding of their significance in the phenomenon of dissociation. Like other variants they may occur gradually after a long process of/

of selection, or they may occur suddenly as a result of some external stimulus or, as it would sometimes seem, they appear for no known reason.

There are a number of unusual colony types which deserve mention as they occur from time to time. Their significance is not yet thoroughly understood.

The "Mucoid Variant": this is a large moist colony of mucoid consistency which has been reported among organisms such as B. anthracis, B. leptosepticus and members of the colon-typhoid-dysentery groups.

The "Phantom Colony": such a type has been described in Friedländer's bacillus, B. anthracis and B. subtilis. It is thin, transparent and difficult to detect except by reflected light.

The "Dwarf Colony": Löhnis and Smith (1923) in their studies of Azotobacter observed "dwarf" colonies which were extremely small and inconspicuous and frequently did not become visible for several weeks. They resulted from the multiplication of conidia. Such forms have been observed in a number of species, e.g. V. cholerae, and may be identical or closely related to the recently described "G" form of B. dysenteriae Shiga (Hadley, Delves and Klimek, 1931). Hadley and his co-workers regard such a form as a visible stage of culture existence lying between an ultramicroscopic/

ultramicroscopic virus and the ordinary culture type. It is viable over long periods of time and may be made to revert to a form possessing all the original characteristics.

Variation occurs within the actual colony, when for example an S form develops an R edge, sector or patch at one side. The two areas usually differ in emulsibility and subcultures may result either in a pure culture of each type of growth, or in a change to another type.

The occurrence of papillae has also been regarded as an indication of dissociation. Incorporation in the medium of carbohydrates or other chemical substances, or simply ageing stimulates their production, and when once present, they either overgrow the parent structure or remain as small protuberances. So far there does not seem to be any satisfactory explanation for this. Replating may give rise to colonies of a type similar or dissimilar to the original, to an organism changed in size or shape or to one characterized by more luxuriant growth. It has been suggested that the occurrence of papillae is due to adverse environmental conditions but since they appear either in the centre or at the edge of a colony in contact with the medium, and may be numerous or scanty under similar external/

external conditions, it seems hardly likely that the environment or products of growth are entirely responsible for their formation. Evidently only certain cells acquire the power of developing secondary colonies in this way and no permanency of the character is guaranteed. Mellon (1922) regards these secondary colonies as the macroscopic counterpart of pleomorphism. Papillae frequently remain viable for longer periods than the parent colony and therefore function as a means of propagating the race under adverse conditions. According to Atkin (1926) pneumococcus papillae developing on an autolysed area are insoluble in bile. They are devoid of autolysin since they show no tendency to dissolve and the cocci retain their Gram staining and do not show degradation like other forms undergoing lysis. Subcultivation on serum-agar slopes results in recovery of the autolytic property and bile solubility.

Variation in Morphology.

As a result of attempts to present some sort of sequence in the phenomenon of dissociation, correlation has been made between the shape and size of the organism and the S and R variants. It has been suggested that cells of "normal" morphology are associated with S/

S, and atypical forms with the R colony. This is true in a number of instances but no general rule can be laid down as there are frequent references to correlation of the S form with rather atypical individual organisms. Reports of colonial variation without change in morphology are not unusual. Peculiarities of bacterial shape and structure have for many years been dismissed without further study as "involution forms" and there has been, and indeed there still is, much hesitation to admit the existence of specialized cells differing in function and appearance from the generally recognized forms. Almquist (1904; 1917) reported the occurrence of conidia and unusual bacillary or spiral forms in cultures of the cholera vibrio and typhoid bacillus. With variation of conditions the latter organism transformed into numerous different rod and coccoid types which showed some degree of constancy but no definite permanency. His findings, however, have not been generally accepted. Mellon and Yost (1926) observed that B. alkaligenes grew in the human body as a large Gram-positive diplococcus which dissociated into bacillary and streptothrix-like stages and also into a stage of mixed morphology, all of which remained true to type. That extreme morphologic/

morphologic variation occurs in other species is evident from reports such as those regarding B. dysenteriae (Twort 1920), Azotobacter (Löhnis and Smith 1923), B. diphtheriae (Smith and Jordan 1930), B. coli (Hausam 1930), B. saccharobutyricus (Cunningham 1931 a, b, c, d) and the pneumococcus (Rakieten 1930). Hort (1920) defined an involution form as one which is undergoing retrogressive or perhaps degenerative changes. He pointed out that there was a wide difference between the faintly stained, imperfectly outlined organism, incapable of multiplying, and the one which was perfectly symmetrical and well stained and yet deviated in some way from the so-called normal. The latter multiply by binary fission, but, to deeply staining organisms which were found from time to time he attributed the ability to reproduce by methods with which the average worker is not usually familiar. Such deeply stained forms have frequently been reported but as a rule any suggestion as to their greater significance is lacking. The work of Cunningham (1931 a, b, c, d) also deserves further mention. A detailed study of morphological and cultural variation was made in twenty strains of B. saccharobutyricus von Klecki. From these, long and short rods, spindle and branched/

branched cells, conidia, microcysts and other forms were obtained. Some of the variants were aerobic and some anaerobic and their degree of stability varied. Cunningham does not arrive at any general conclusion as to the nature of the factors which control the development of the various phases of this organism. He does, however, consider it improbable that variation in environmental conditions is of primary importance in regulating the appearance of transformations and suggests that they may be associated with some stage in the life history of the organism which is only recognizable with difficulty or is as yet unrecognized.

Variation in capsule formation has received considerable attention in the case of the pneumococcus, pneumobacillus and anthrax bacillus. Fiscoeder (1909) believed that the capsule of the anthrax bacillus formed no protection against body defences. This, however, was not supported by the later work of Preisz (1911) who reported that the more stable and persistent the capsule the more virulent was the organism for mice. It may be concluded from studies of the pneumococcus (Heidelberger and Avery 1923; Avery and Heidelberger 1925; Dawson, 1928) and the pneumobacillus (Julianelle, 1926) that the capsular material is composed of a complex/

complex carbohydrate which is closely associated with type-specificity. The R variant generally lacks this capsular material and also type-specificity.

The occurrence of asporogenous races of B. anthracis has been a subject of particular interest ever since Pasteur advocated the use of anti-anthrax vaccines. Both sporogenous and asporogenous variants were found in laboratory cultures examined by Eisenberg (1912a). The former could be obtained in pure culture by heating the mixed strain to 70-90° C. and the latter selected out by transfers in milk at 12-30 hour intervals. It was also possible to transform a sporogenous into an asporogenous race by passage on glycerine-agar. Nungester (1927; 1929) reported seven different varieties of anthrax colonies all of which were sporogenic although in the case of the two mucoid types this character was greatly reduced. S and R colonies of B. subtilis (Soule, 1928) produced spores equally readily; but in B. mesentericus-vulgatus (Gee, 1927) the S variant lacked this property. It was found possible to cause the R → S reversion but when inducing the opposite transformation, although the power to form spores was regained, the colonies were not identical with those of the original R. Exospores, which, when fully developed are indistinguishable from endospores/

endospores, have been observed in strains of B. saccharobutyricus (Cunningham, 1931c). It is possible that too general an acceptance of the belief that intra-cellular spore formation is the only type encountered in bacteria has hindered the observation of the extra-cellular forms in other species.

Finally, the existence of filterable organisms must be considered. That ultramicroscopic viruses cause disease is well known, but the occurrence of filter-passing elements derived from non-filterable bacteria still remains a subject of controversy. Such forms have been reported in the case of B. tuberculosis (Hauduroy and Vaudremer, 1923; Vaudremer, 1923; Valtis, 1924) and certain intestinal organisms. Hauduroy (1924a,b) obtained minute elements after filtration of B. dysenteriae cultures treated with phage. These reproduced similar forms or returned to the normal type. They usually differed in fermentation reactions from the original cultures. Löhnis and Smith (1923) and others believed that such minute bodies represent a stage in the life cycle of the respective organism. Recently Hadley, Delves and Klimek (1931) described a filterable form of B. dysenteriae (Shiga), the visible elements of which were different in most of their biological and morphological/

morphological characteristics from the "normal"; they had a virus-like phase in their existence when they were not visible either as colonies or as individual organisms. The authors remark that they showed the existence of similar filterable bodies for ten other bacterial species, but details of these experiments were not given. In the case of the typhoid bacillus an ultramicroscopic form has been reported by Friedberger (1927). Post-mortem material from a case of typhoid was injected into guinea-pigs, and transferred in series by injecting an emulsion of organs and blood from one animal to another. The virus was not visible, nor was it possible to cultivate it on ordinary media. There was, however, evidence of its existence since guinea-pigs developed pyrexia and were immune to multiple lethal doses of the virulent typhoid bacillus. Furthermore, organs and serum taken from a guinea-pig during the pyrexial phase and injected intravenously into rabbits produced anti-typhoid agglutinins.

Variation in Fermentation Properties.

For many years the ability of certain organisms to ferment carbohydrates has been used as a method of classification, but exceptions to the recognized reactions are not infrequent. The variation may involve/

involve the acquisition of ability to ferment some substance with the production of acid and gas, or, conversely, it may involve the loss of this power. On the other hand, the difference may be one of degree only, the speed of the reaction being markedly more rapid in one case than in another. The sudden appearance of organisms which have lost or gained certain of these characters may be seen in the case of papillae showing a different reaction from the parent colony when growing on a medium containing the particular carbohydrate under investigation and a suitable indicator. Neisser (1906) and Massini (1907) first observed this in a strain which they named B. coli mutabile and which they cultivated on lactose medium. On subculturing, the acid papillae remained true to type and the parent structure gave both fermenting and non-fermenting colonies. Reactions analagous to this have been described for other species by a number of investigators (Müller, 1911; Penfold, 1911a; Baerthlein, 1912; Braun and Löwenstein, 1923; Lagrange, 1928) and, conversely, the occurrence of white non-fermenting papillae on iso-dulcitate fermenting colonies has been observed in the case of B. dysenteriae (Ledingham, 1918). It is possible to induce a gradual change of fermentation properties by repeated transfer in/

in fluid or on solid medium and in this way to change the characteristics entirely or simply to increase the rate of the reaction. Penfold obtained a strain of B. typhosus which produced acid in dulcitate peptone water in one day instead of requiring ten days as at the beginning of the experiment. This author also pointed out that the permanency of the newly acquired character varies inversely as the time required for its selection of training. Goodman (1908) cultivated B. diphtheriae in dextrose broth selecting for sub-cultivation those tubes showing the greatest and the least amount of acid. After thirty-six such selective transfers one race gave an extremely acid reaction and the other a much lower degree of acidity than in the original culture.

Variation in gas production is not an unusual feature of carbohydrate fermentation. Freshly isolated strains of organisms, which normally would produce gas, occasionally lack this property in primary culture but develop it after repeated subcultivation. Variants failing to produce gas were obtained by growing strains of coliform organisms on a medium containing monochloroacetic acid agar (Penfold, 1911b). To investigate the possibility of transforming an aerogenes into an/

an anaerogenes variety, Mackie (1921) cultivated several strains of B. coli on this medium and found that the resulting variants simulated those of Penfold in failing to produce gas from glucose, but differed from them in producing it from lactose. It was not found possible to select an anaerogenes from an aerogenes B. coli type.

Variations are recorded in studies of many other organisms, and some authors suggest that as a result of this variability such fermentation tests can only have a limited application as a means of differentiation. While, therefore, the possibility of variation must be considered when identifying organisms it is not generally believed that such exceptions invalidate the tests. In fact, the ability of B. typhosus to form papillae on an iso-dulcitate medium is considered by Müller (1911) and by Penfold (1912) a reaction of sufficient specificity to be included in the classification of the organism.

Variation in Pigment Formation.

Among staphylococci in which pigment formation plays an important part in classification not only are shades of colour intermediate between those of aureus and albus to be found but also some or all of the types may/

may be interchangeable (Neumann, 1897; Baerthlein, 1918; Bigger, Boland and O'Meara, 1927). Dudgeon (1907) believed sufficient evidence existed that all staphylococci are one species. Rettger and Sherrick (1911) by a process of repeated selection of pigmented and non-pigmented growth from agar slope cultures of B. prodigiosus obtained two races, the one bright red and the other lacking the characteristic pigment. The latter was the more stable of the two. In a study of several strains of this species Eisenberg (1914) induced variants ranging from dark red to colourless forms, and associated with these were colonies of different consistency. As a result of varying the medium fluorescin or pyocyanin may be produced irrespective of each other from a strain of B. pyocyaneus which originally possessed the property of forming both these pigments (Sullivan, 1906). Under the influence of a lytic agent Fejgin (1924) obtained yellow variants from B. proteus X19. They appeared spontaneously and lacked the fermentation properties of the original organism.

Variation/

Variation in the Decomposition of Proteins.

Variation in the decomposition of protein has been frequently studied in regard to the ability or lack of ability to break down gelatine, serum or casein. Indole production is also a variable reaction. The R variant of B. subtilis (Soule, 1927) and the opaque colony of V. cholerae (Balteanu, 1926) both liquified gelatine more slowly than the original cultures. In the latter organism there was also a decrease in indole production. Diminished proteolytic ability is associated with the R form of B. pyocyaneus (Hadley, 1927), the "dunkle form" of the cholera vibrio (Eisenberg, 1912b) and colonies of B. proteus (Firtsch, 1888; Baerthlein, 1918) which are now regarded as identical with the O form of the organism. By transferring non-indole producing strains of B. typhosus in an appropriate medium Peckham (1897) induced variants which had acquired the property. They frequently appeared after the third passage.

Variation of Growth in Fluid Medium.

Different types of growth in broth or fluid media is one of the most striking peculiarities of bacterial variants, the S frequently causing a uniform turbidity throughout the medium, with or without a compact sediment/

sediment; the R a pellicle and granular sediment with a clear fluid. Two such types of growth have been described in many organisms among which are those of the typhoid-dysentery groups (Arkwright, 1920, 1921), the bacillus of rabbit septicaemia (DeKruif, 1921, 1922) and the tubercle bacillus (Petroff, 1927). It must be remembered, however, that there are many exceptions and that a granular growth may be associated with S colonies as in the case of B. diphtheriae and streptococci.

When S organisms are emulsified in physiological salt solution a uniform suspension results, while the R growth is broken up only with difficulty. This peculiarity of R organisms is known as spontaneous agglutination, and was observed in cultures of the typhoid bacillus long before the phenomenon was associated with dissociation (Nicolle, 1898; Savage, 1901). It was noticed often with old cultures or those cultivated for long periods of time on artificial media, in which, it is now known, R forms frequently predominate. Beniasch (1911) attributed clumping to hydrogen-ion concentration and Arkwright (1921) found that it was possible to stabilize suspensions of R organisms in 0.42, 0.2 or 0.1 per cent. saline as well as in distilled water. This, however, is not possible in the case of/
of/

of all organisms, some of which in the normal form, such as B. salmonicida, agglutinate even in distilled water. White (1928) believes that the stability of a bacterial suspension in an aqueous medium depends on the dominance of the hydrophile factors of the bacterial surface over the hydrophobe factors, the constituent of which in fresh cultures is an alcohol- and chloroform-soluble lipoid. In R cultures this lipoid element predominates in the hydrophile properties of the basal proteins and agglutination occurs in a saline medium. In S cultures the presence of the soluble specific substance which is insensitive to electrolytes acts as a stabilizing agent and agglutination does not occur. In the case of agglutination of S cultures as in B. salmonicida White believes this to be due to the large quantity of the "lipoid" component or great activity of the hydrophobe factor.

Variation in Antigenic Properties.

At one time identification of variants was established by means of agglutination reactions, the variants being tested with stock antisera for the so-called "normal" type. Within recent years, however, it has been shown that variation in antigenic structure itself is by no means uncommon. At first inagglutin-
:able/

inagglutinable strains of B. typhosus were reported without any satisfactory explanation of their occurrence. Later, emphasis was placed on lack of motility in these strains and then the presence or absence of flagella was correlated with changes in antigenic structure.

At the present time three main types of antigenic variation are recognized: (1) H-O variation, (2) S-R variation, (3) specific phase-non-specific phase variation. Numerous intermediate forms occur between the extremes (except possibly in the case of phase variants), but because of their mixed characters and instability they have not been studied in detail.

H-O Variation.

In a motile species the organism possesses two antigens, one associated with the flagella, the other with the body of the organism. Loss of the flagella, as a result of spontaneous or artificial dissociation, results in a variant form which lacks the flagellar or H antigen. The nomenclature arose as the result of the work of Weil and Felix (1917) on B. proteus, the respective antigens being associated with the H or spreading and O or restricted colonies. These authors found that serum from a case of typhus fever agglutinated/
:ed/

agglutinated the so-called "X" strains (X2 and X19) of B. proteus in small firm flakes, the reaction taking place slowly. An immune serum obtained by injections of either of these strains - but particularly with X19 - agglutinated the organism used to produce it rapidly and in large loose floccules. It was found that patient's serum or immune serum for the O variant contained only one agglutinin which reacted specifically in small flakes with the homologous organism, while immune serum possessed not only the O agglutinin, but also a non-specific large flaking antibody which agglutinated heterologous as well as homologous strains. The H antigen was thermolabile and the O thermostable, the latter withstanding prolonged heating at 100° C. On the other hand the H agglutinin was heat-stable while that of the O serum was destroyed at 60° C.

The work of Weil and Felix on B. proteus has been confirmed and extended by other investigators (Braun and Salomon, 1918, 1919; Braun and Nodake, 1924; and others). Similar antigenic variation in motile species has been recorded before this time, but not until the observations of Weil and Felix were published did the significance of the earlier work become evident. Since then the H and O antigenic constituents have been studied/

studied in organisms of the typhoid and salmonella groups (Weil and Felix, 1920; Furth, 1923; Goyle, 1926, 1927; and others) and other organisms such as V. cholerae (Balteanu, 1926) and certain anaerobes (Felix and Robertson, 1928).

The appearance of R variants complicates the apparent simplicity of H-O variation.

S-R Variation.

When an R variant arises in a motile species there is apparently a change in the somatic antigen without alteration of the flagellar component. Arkwright (1920, 1921) correlated strains which were easily emulsified in physiological salt solution and S colonies with one form of antigen and spontaneously agglutinating R colonies with another. Working with a non-motile organism (B. dysenteriae) he found that both the antigens were of the heat-stable somatic type and were agglutinated by stock antiserum to the same titre. With sera prepared against the S and R variants respectively, each agglutinated its specific strain but there was little cross agglutination.

The similarity of non-motile S and O variants, both of which are devoid of heat-labile antigen and are characterized by the presence of the thermostable component/

component, has been observed by several investigators (Goyle, 1926, 1927; Arkwright, 1926).

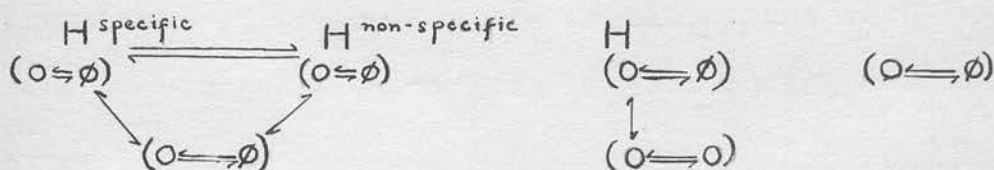
In the case of capsulated organisms the same somatic antigen is common to both S and R variants. A soluble specific substance, associated with the capsular material, has been extracted from the pneumococcus (Avery and Heidelberger, 1923, 1925; Heidelberger and Avery, 1923, 1924; Avery and Morgan, 1925) and the pneumobacillus (Julianelle, 1926, a, b and c, 1928), and although this reacts with the homologous serum of the type from which it is obtained it is not actually antigenic per se, but only in association with another fraction of the cell. If the substance is removed artificially or is naturally absent as in the case of the R variant, the organism is found to have lost its type specificity and shows only group specificity. A soluble specific substance probably similar in nature to that of the pneumococcus and pneumobacillus has been obtained from streptococci (Lancefield, 1928), B. coli (Dulaney, 1928) and other organisms.

Specific/

Specific Phase — Non-Specific Phase Variation.

A further complication in the study of antigenic variation was added by the work of Andrewes (1922, 1925) on certain members of the Salmonella group. Some organisms showed type specificity and others marked co-agglutination but there was no relationship between these antigenic variants and S and R colonies, since all were smooth in structure and readily emulsified in saline. The types were reversible and belonged to either the specific or non-specific phase. Intermediate mixed types were rare. It was found that a group serum of one strain, besides agglutinating the group variants of homologous strains, agglutinated the group variants of heterologous strains to a relatively high titre but it had only slight effect on its own type in the specific phase and no effect on heterologous strains in the specific phase.

As a result of this and other work the Salmonella organisms have been classified as "Diphasic", "Monophasic" and "Meta" types. The reactions have been represented diagrammatically thus:-



(White - A System of Bacteriology.)

Diphasic organisms exhibit marked interchangeability of H specific and H non-specific antigens and at the same time show S-R variation (represented by O-Ø).

In monophasic strains, besides S-R variation, the H antigen occurs also but it is in either the specific or the non-specific phase and the two are not interchangeable.

Finally, the non-motile strains, Salmonella pullorum and Salmonella gallinarum have been designated Meta-Salmonella types and they undergo only S-R variation.

Variation in Virulence and Pathogenicity.

Frequently microorganisms, after repeated subculture on artificial media, are found to lose their virulence. As a rule subsequent passage through susceptible animals or growth on a medium enriched with some substance such as serum or blood causes a restoration of the lost characteristic. Loss of virulence in a culture has, for many years, been attributed to a general decline in virulence of the individual organisms, but it now seems more probable that cells varying in virulence occur side by side in the same culture/

culture. This is borne out by numerous records of variation among colonies of a particular strain. Correlation between S and R and virulent and avirulent forms respectively has been established in the case of organisms such as B. lepi-septicus (DeKruif, 1921, 1922), the pneumococcus (Reimann, 1925; Dawson and Avery, 1928; Dawson, 1928), the pneumobacillus (Julianelle, 1926) and organisms of the coli-typhoid group (Topley and Ayrton, 1924; Goyle, 1926, Ibrahim and Schütze, 1928). Unfortunately too many exceptions occur to permit a general acceptance of this correlation. S variants of streptococci were found to be the more virulent Cowan (1923) and Dutton (1928) but similar results were not obtained by Todd (1927, 1928, 1930), who isolated an R form from the blood of a patient suffering from a severe streptococcal infection, and degraded it to an S avirulent type. Eagles (1928) confirmed the work of Cowan (1923) when using a particular strain but with another strain the R proved to be the more virulent.

In the case of pneumococci Jacobson and Falk (1927) pointed out that strains differing in virulence were not necessarily separable into S and R categories. Yu (1930), in an investigation of B. diphtheriae strains isolated/

isolated during the active and convalescent stages of the disease, found that the R variant was always avirulent and atoxic but the S form was frequently, though not always, virulent. Such clear-cut results have not often been obtained with this organism and notably opposed to them is the recent work of Anderson, Happold, McLeod and Thomson (1931), who associated a type producing a large rough or "daisy head" colony with severe toxic cases of diphtheria and a smooth type with milder cases. In the latter investigation a special blood-agar medium was used, while Yü's examinations were made on ordinary blood-agar. It seems evident, therefore, that although in some species virulence may be associated with the S colonies, this is by no means a uniform characteristic and exceptions may occur even among strains of the same species.

Close association of toxin production and S variants is also suggested (Yü, 1930; Goyle, 1926; Ibrahim and Schütze, 1928) but again exceptions are apparent, atoxic strains of tetanus bacilli occurring without change in morphological or cultural characteristics (Fildes, 1927) and toxic filtrates of haemolytic streptococci from matt and glossy colonies showing approximately/

approximately equal toxigenicity when the toxins have been tested with the Dick cutaneous reaction (Todd and Lancefield, 1928). DeKruif (1922) in his studies of B. lepi-septicus observed that the S organism owed its greater invasive power at least in part to its anti-phagocytic property, and similarly, a decrease in virulence and increase in phagocytability after growth in homologous immune serum were associated in strains of pneumococci (Stryker, 1916) with dry, isolated, brownish colonies - presumably R types - instead of with the confluent growth usually appearing on blood-agar.

Modification in virulence of organisms of the same strain naturally suggests the possibility of immunizing with the avirulent form and thus protecting against a subsequent infection with a virulent type. The S form of B. paratyphosus A (Arkwright, 1926) proved to be a more efficient vaccine than the R form; the latter, however, was not entirely devoid of value. The motile and non-motile S variants were equally effective. Animals surviving infection with the relatively avirulent G type of B. lepi-septicus proved resistant to multiple lethal doses of the highly virulent D organism (DeKruif, 1921a). Cowan (1923) obtained similar results/

results with streptococci but Lancefield and Todd (1928) found that only in the case of matt organisms (the virulent forms of the strains they studied) was an immunity, either active or passive, produced against infection with the homologous strain. Immunization of experimental animals with avirulent pneumococci did not lead to the development of protective antibodies for the virulent strain (Amoss, 1925). It is evident, from the lack of uniformity in results, that no practical application of this method of immunizing is likely at present. There is, however, one notable exception and that is the immunization of infants, and recently to some extent of adults, with the attenuated bovine tubercle bacillus of Calmette and Guérin (B.C.G.) Since growth of an R organism in its homologous immune serum causes the R-S transformation in vitro, there seems no reason why such a reversion should not occur in vivo and therefore, theoretically at least, the safety of immunization with living R organisms is a matter of doubt.

The/

The Stability and Reversibility of Bacterial Variants.

Although frequent references point to the stability of S, R and sometimes I variants, there are equally numerous instances of instability in these types. It is possible that such forms have not been dissociated to a "stable phase" but so far there is no definite evidence to suggest that, from the point of view of colony structure at least, the organism goes through a cycle in the various stages of which marked stability or instability is exhibited. Undoubtedly external stimuli influence the transformation from one form to another, but the actual manner in which they effect this is not known, and though the inconsistent results may point to strain peculiarity it seems much more likely that some other mechanism or combination of circumstances is responsible in whole or in part for the phenomenon.

The work of Kauffmann (1928) on type specificity of B. paratyphosus is of interest since subcultivation of specific variants resulted in 95 per cent. of the same type and 5 per cent. of group variants. Similarly, the latter when subcultured only gave 5 per cent. of group colonies, the remainder being in the specific phase. Records of such uniformity are unusual. In most/

most organisms reversion of R → S or of group → specific is to the type from which the variant was derived but Griffith (1928) found that by inoculating a mouse with an attenuated R strain of pneumococcus of one type plus a large dose of another type killed by heating to 60°C. a virulent S of the heated culture type could be obtained. Other experiments suggest the possibility of complete interchangeability of species, haemolytic and viridans streptococci being interchangeable, and passing through stages showing variable degrees of haemolysis and greenish discolouration (Zdansky, 1928). All types were isolated from the blood of a patient suffering from endocarditis and were made to revert to S. haemolyticus. It is possible that the appearance of these variants in vivo is due to dissociation as a result of antibody production by the infecting organism.. Morgenroth, Schnitzer and Berger (1926) maintained that they could change pneumococci to streptococci of the viridans type and then to haemolytic types and that the transformation was reversible.

Just how far microorganisms may vary or interchange is uncertain, but it seems possible that races which have received specific names may really be variants of the same strain. For instance, Soule (1928) believes/

believes that B. cereus of Chester may be identical with the R form of B. subtilis, and Löhnis and Smith (1923) who observed relatively stable types of Azotobacter found that irregular, fungoid cells producing yellow or orange pigment were identical with Mycobacterium luteum Söhngen and Mycobacterium lacticola Lehmann and Neumann, respectively.

Theories of Microbic Dissociation.

When the phenomenon first began to interest bacteriologists they frequently referred to it as a "mutation". Considering that the variants were not stable even when maintained under certain conditions, the change could not be regarded as a true mutation and the term was soon replaced by that of "dissociation".

"Degeneration of cultures", "degradation of cells" and "disease of bacteria" were among the theories put forward in explanation. All were based on the monomorphic conception of the nature of bacteria and have been discarded in view of the fact that the variant is as readily cultivated as the undissociated form and gives no evidence of degradation.

A more possible hypothesis is that which suggests that bacterial dissociation is an adaptive reaction. New or unfavourable conditions are usually accompanied by/

by dissociation, the resulting R form being resistant to these conditions, and capable of active growth. On the other hand, dissociation has been observed in young cultures on a rich medium, and it seems possible that since the reaction may be associated with a rate of growth slower than the optimum (as, for instance, when an organism is cultivated on a "starvation" medium) a similar variation might be induced by overstimulation of growth, resulting from cultivation of the organism on an excessively rich medium.

Hadley (1927) puts forward a hypothesis which, he admits, rests on a basis none too secure in facts, but has, nevertheless, sufficient truth in it to make it worthy of mention. Superficially microbic dissociation involves the partial or complete elimination by autolytic or transformatory processes of the S type, when conditions become unfavourable for continuance of the same type of growth, and is accompanied by a generation of new bacterial forms better qualified to perpetuate the strain. Fundamentally, he says, it may be regarded as an adaptive reaction made possible through the intervention of a special type of reproductive mechanism involving nuclear reconstruction, which is effected by the conjugation of certain cells and/

and the production of zygospores. A process of division or budding of these cells results in the formation of new elements - cocci, granular bodies or filterable forms, the type which becomes stabilized depending on the degree of reaction and the selective nature of the environment. The R form is therefore, the completely or partially stabilized form resulting from the germination of special cell structures. The S types surviving dissociation are the remnants of the original culture which have not entered upon the modified reproductive process.

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From the foregoing brief outline it is evident that a microorganism may undergo variation in the majority, possibly in all, of its characters and reactions. While admitting, therefore, the occurrence of variation, a review of the literature and the results recorded in the following pages, suggest very strongly that recently undue emphasis has been placed on the so-called S and R variants, and as a consequence insufficient attention has been paid to other types. Such emphasis has been of some value, since, by stressing the presence of certain forms and suggesting the possibility/

possibility of a definite sequence in dissociation interest in the subject has been aroused and the monomorphic conception of microorganisms disproved; but, in ignoring the more unstable or unusual forms, an explanation of the phenomenon may be overlooked.

This subject will, however, be discussed in greater detail after a presentation of the results obtained in the study of acid-fast and diphtheroid organisms.

METHODS OF STUDYING DISSOCIATION

Dissociation has been usually studied by the use of mass cultures or by the selection of colony variants. With respect to the latter, the work of various bacteriologists has frequently been criticized from the point of view that investigations were not carried out with single cell cultures and therefore peculiarities of even well isolated colonies might be explained by their origin from more than one organism. Although this criticism is perfectly justified a comparison of the results obtained with and without the use of single cell cultures shows no marked differences.

Broadly speaking, dissociation may be induced by four main types of stimuli: (1) physical, (2) chemical, (3) serological and (4) bacteriophage influence.

The following gives a brief outline of these methods:-

Physical Stimuli.

Temperature.

Increased temperature in the case of the anthrax bacillus (Roux, 1890; Preisz, 1911) caused variation in virulence, spore formation and capsule formation.

Avirulent/

Avirulent "mutants" were reported by Almquist (1917) in B. typhosus after heating to 45°C. Subnormal temperatures are not usually considered so likely to affect variation but in the streptococci growth at room temperature caused granular, spontaneously agglutinating strains to become diffuse (Shibley, 1924). A similar observation was made for diphtheroid bacilli and was associated with morphological and staining changes (Mellon, 1922).

Ageing and Dessication.

Changes of various characters in old stock cultures have been recognized for many years. On plates an R edge or papillae may arise from an S variant. In part these variations are possibly due to ageing or change of temperature. They may also, however, be affected by dessication or by excessive moisture.

Volume of Medium and Repeated Transference.

Both with regard to solid and liquid media the quantity employed plays an important part in dissociation. The necessity for large amounts of solid media has been stressed generally with a view to preventing too rapid dehydration. With liquid media Soule (1928) found that the S → R transference in B./

B. subtilis occurred after subcultivation in 300 c.c. volumes but not in 5 c.c. amounts. Similar results were obtained in producing an actively fermenting race of B. typhosus (Penfold, 1913). Even when the volume of the inoculum was proportional to the volume of the medium, the fermenting race arose more quickly in the large quantity of broth. Penfold concluded that the more effective selection from a large population is due mainly to the presence of more extreme variants from the mean and that the explanation is not that in the large cultures a few more generations are required to produce the same population per c.c.

The necessity for repeated transference to obtain a stable variant requires emphasis as all too frequently only partially stabilized types are regarded as constant forms, with resulting confusion to the literature. Two hundred transfers were required to obtain stable R variants of B. subtilis (Soule, 1928) and an even greater number have been used by Calmette and Guérin to attenuate a bovine tubercle bacillus which they isolated in 1908 (Brit.Med.J., 1931, i, 1070). The majority of investigators now consider this strain is avirulent but its colony forms cannot be regarded as stabilized except on glycerine-bile-potato, the medium on which it has been/

been cultivated. Raw (1926) also found it possible to attenuate a tubercle bacillus by subcultivation on glycerine-egg and glycerine-potato alternately.

Hydrogen-Ion Concentration.

As important as the volume of medium is the pH. An alkaline reaction is usually regarded as favourable to the S \rightarrow R dissociation. In the case of B. subtilis (Soule, 1928) a neutral or slightly acid reaction seemed more suitable. Growth of the organisms in liquid medium by changing the pH may assist the transformation as Koser and Styron (1930) found with B. dysenteriae, no variation occurring till a change from the alkaline to the acid side took place. The pH of a medium resulting from growth in it of dissociates of the same strain may be different for the S and R variants, the S form of B. whitmori (Nicholls, 1930) causing marked alkalinity and the R form only slight alkalinity. Similarly, with variants of B.C.G. Petroff, Branch and Steenken (1929) observed that the R form caused a change in the reaction of the medium from the acid to the alkaline side, whereas the S form increased the acidity.

Oxygen/

Oxygen Tension.

Oxygen tension also influences dissociation. Either low tension or between 40 per cent. and 60 per cent. increased the S \rightarrow R transference but not the R \rightarrow S in the case of B. subtilis (Soule, 1928). 80 to 100 per cent. had a stabilizing influence. Working with B. leipsepticus Webster and Burn (1926) isolated a variant which multiplied with extreme rapidity in a medium of relatively low oxygen tension. The ability of partial tension organisms such as B. abortus (Smith, 1924) or the gonococcus (Wherry and Oliver, 1916) to become accustomed to ordinary atmospheric conditions is also suggestive of dissociation.

Chemical Stimuli.

Dyes, antiseptics and other chemical substances are all known to produce a marked effect on microorganisms. The influence of carbohydrates on the formation of papillae has already been mentioned. The changes resulting from the incorporation of these substances in a medium are not purely superficial but may involve relatively permanent variants of altered morphologic, cultural and antigenic structure. Phenol, a particularly useful incitant, has been employed to induce /

induce asporogenous races of B. anthracis (Roux, 1890) and variants of proteus X19 (Braun and Schaeffer, 1919) and certain intestinal organisms (Hoder and Suzuki, 1927). Koser and Styron (1930) investigated the effect of peptone in inducing dissociation of B. dysenteriae and found a 5-10 per cent. concentration of Bacto peptone to be the most favourable. Different brands of peptone gave different results.

Lack of nutrient material or "starvation" methods have also proved effective.

Serological Stimuli.

A rapid and, as a rule, effective method of inducing dissociation is to cultivate the organism in its homologous immune serum, 10 per cent. of the serum being the most suitable dilution. This has been amply proved in the case of many organisms. The S → R transference usually takes place more readily than the R → S as shown by the experiments of Arkwright and Pitt (1929). Normal serum may assist dissociation but usually it acts in a manner similar to animal passage in stabilizing the virulent form. When, however, it does stimulate dissociation it is possible that natural antibodies present in the serum may play some part in the/

the phenomenon. This may explain the isolation of avirulent variants from chronic cases or carriers of diphtheria (Yü, 1930), typhoid (Olitski, 1928) and other diseases.

Heterologous immune serum does not produce variants as readily as homologous serum.

Variation Induced by the Action of Bacteriophage.

Some evidence exists to show that bacteriophage may cause variation. The mechanism of the reaction, however, remains controversial, since the true nature of phage is as yet unknown. The dissociates frequently resemble those induced by other means, changes being observed both in the form and the antigenic structure of, for example, various intestinal organisms (Hoder and Suzuki, 1927). Individual cells in a culture vary in their degree of susceptibility to phage, even in S and R cultures where the former is usually considered the more sensitive (Gratia, 1921; Arkwright, 1924). Cells surviving lysis may form secondary colonies on the lysed area or even overgrow it and they may transmit the lytic principle without themselves actually undergoing lysis.

ACID-FAST BACILLI

Although a study of microbial dissociation has been made in many species, the Mycobacteria have received but little attention from the point of view of colony structure and associated virulence. The morphological variation of these organisms has, however, been studied in detail and an extensive review of the literature has been made by Kahn (1929). No doubt the slow rate of growth of some of the organisms and the difficulties involved in incubating plate cultures of suitable media for several months, without dehydration or contamination, has been largely responsible for the lack of attention to colony form.

In 1913 Baerthlein and Toyoda obtained moist, glistening and dry, friable colonies from a strain of frog tubercle bacillus. The moist colony was composed of long, slender organisms, the dry variant, of shorter cells. When grown on weakly alkaline glycerine-agar or when passed through animals the variants remained constant. There was no marked difference in virulence when relatively large doses were given. This organism, however, grows more rapidly than either the human or the bovine tubercle bacillus and it was not until/

until Petroff (1927a) evolved a suitable technique for studying such slow growing strains that interest in the dissociation of acid-fast organisms was aroused.

Petroff and his co-workers found that in human, avian, bovine and attenuated bovine (B.C.G.) strains of the tubercle bacillus several types of colonies were present. By the addition of 0.25 per cent. sodium taurocholate to the gentian-violet-egg medium on which the organisms were cultivated, a marked differentiation of types was obtained. As has already been mentioned the colonies of acid-fast organisms are particularly complex and only in certain instances was the symbol S (connoting a smooth, round, entire colony) applicable. Differences in the ease with which the variants could be emulsified in physiological salt solution, their growth in fluid media and their virulence indicated types which corresponded to S and R dissociates in other species.

Outline of the Investigation.

Reports of dissociation in acid-fast organisms - particularly in the case of the attenuated strain of Calmette and Guérin (B.C.G.) - show marked discrepancies. A further study has been made, not only of human and bovine strains of the tubercle bacillus, but also of some/

some more rapidly growing acid-fast organisms, in order to determine whether colony types maintain their characters in subsequent generations and exhibit any variation in virulence when injected into susceptible animals.

Technique.

Unless otherwise stated the technique described in detail by Petroff and Steenken (1930) was employed throughout the work with acid-fast organisms.

The medium consisted of chopped ox heart infused in a 15 per cent. solution of glycerine in water. After twenty-four hours in the refrigerator, to one part of meat juice and two parts of egg, an alcoholic gentian-violet solution was added to make a final dilution of 1:30,000. The dye made a satisfactory background for the observation and photography of white colonies and also, to some extent, inhibited the growth of contaminants. Because of the long periods of incubation the petri dishes required a deep layer of medium and were sealed with tightly fitting rubber bands to prevent dessication and contamination.

Growth from isolated colonies was emulsified in saline adjusted to pH 7.8, the suspension filtered twice through two layers of Whatman's No. 5 filter paper/

paper and diluted from ten to twenty times according to the organism. A large number of plates was then inoculated with two or three drops of the diluted filtrate.

For examination of colonies a Leitz Binocular plate culture microscope was used.

For pathogenicity experiments with strains of tubercle bacillus, rabbits and guinea-pigs were used. Before inoculation all animals received 0.2 c.c. of a 1:1,000 dilution of Koch's Old Tuberculin. In no case did a positive reaction occur in this preliminary test. As a rule tuberculin tests were made weekly until a positive reaction occurred. An autopsy was performed on every animal and attempts were made to demonstrate acid-fast organisms in internal organs and lesions. Where necessary, histological examination of lungs, liver, spleen, kidney, brain and glands, was carried out.

THE BOVINE TUBERCLE BACILLUS

The S variant of the bovine tubercle bacillus (Petroff, 1927 a,b; Petroff and Steenken, 1929, 1930) has been described as round, smooth, opaque and easily emulsified. On replating, it developed a round, flat colony. The R type was large, flat, moist and opaque. It was emulsified only with difficulty and on subculture developed flat colonies. Injection into guinea-pigs of a few S bacilli produced progressive tuberculosis and death, while the R organisms, although leading to tubercular changes were less virulent and the lesions which they produced eventually healed. Tzeknovitzer (1930) was unable to confirm these results.

A further study of dissociation and pathogenicity of this organism has been made.

Dissociation Experiments.

For these experiments a strain of bovine tubercle bacillus was used which had been cultivated in the laboratory for nine years and had been grown continuously on Dorset's egg medium.

It was plated, according to the technique already described, on gentian-violet-egg medium and the following/



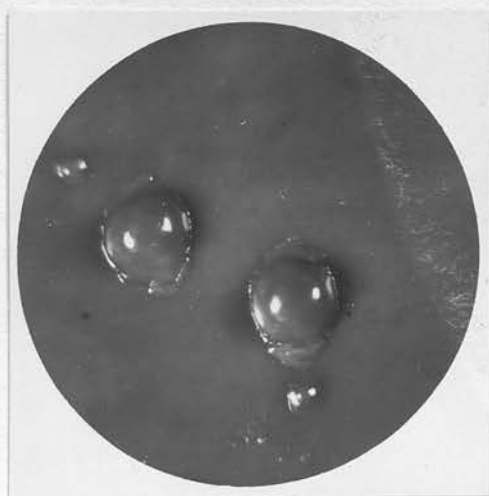
THE BOVINE TUBERCLE BACILLUS

Fig. 1. Variant I.
56 days' growth. x 10.

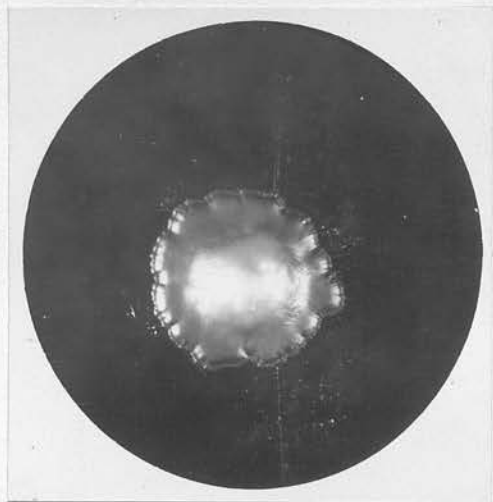


Fig. 2. Variant I.
135 days' growth. x 10.



Fig. 3. variant I showing
papillae. 153 days' growth.
x 10.

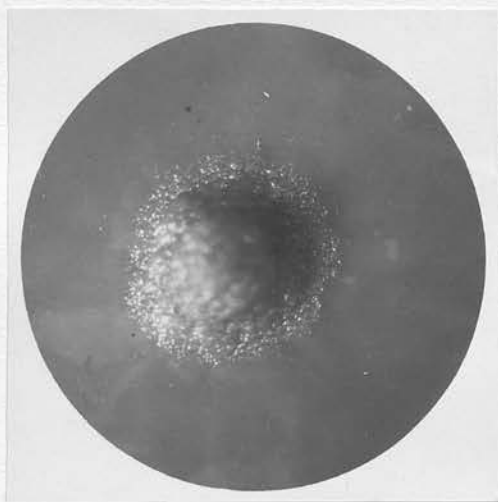


Fig. 4. Variant II.
48 days' growth. x 10.

following two distinct variants developed:

Variant I. The young colony was round, opaque, moist, convex and glistening and was readily emulsified in normal saline (Fig.1). As the colony aged an opaque, glistening border developed and the edge became slightly irregular. The surface, although still glistening and smooth, appeared somewhat undulating (Fig.2). Papillae formation on this type was not unusual (Fig.3).

Variant II. This was a larger colony than Variant I, with a rough surface which gave the appearance of numerous closely packed papillae. The centre was raised and sloped down to a thin, rough periphery, slightly irregular at the edge and through which the colour of the medium showed. To the naked eye it appeared rather dry and of a light yellow colour. The growth was emulsified only with difficulty (Fig. 4). As the colony aged the periphery extended slightly and became thicker and more glistening (Fig. 5).

In order to test the stability of the types they were both replated. Variant I gave the following results:-

Variant IA. On the majority of plates appeared large, moist, opaque colonies. The surface had a glistening beaded appearance and the periphery was smoother than the centre. Several were characterized by a large central coil. They were fairly readily emulsified (Fig. 6; Fig. 8). With prolonged incubation the centre of the colony did not show any marked change but a narrow glistening periphery was formed (Fig.7).

Besides Variant IA several other types of colonies were seen on the same batch of plates.

Variant/

THE BOVINE TUBERCLE BACILLUS

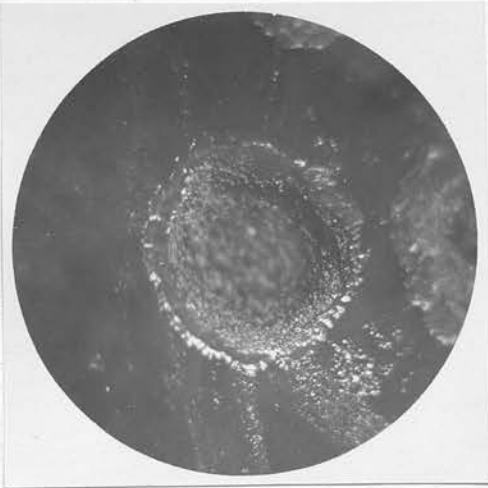


Fig. 5. Variant II.
127 days' growth. x 10.

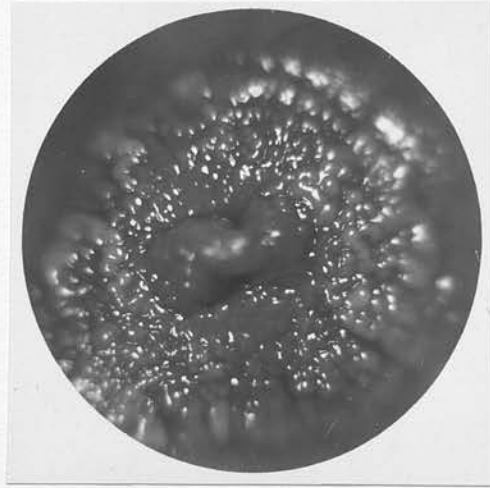


Fig. 6. Variant IA.
73 days' growth. x 10.

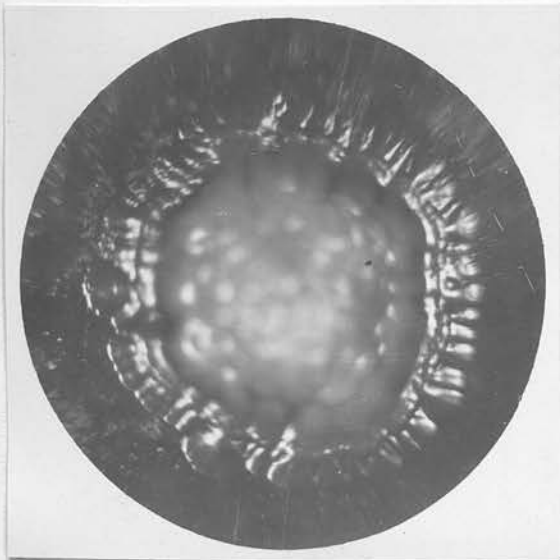


Fig. 7. Variant IA.
245 days' growth. x 10.

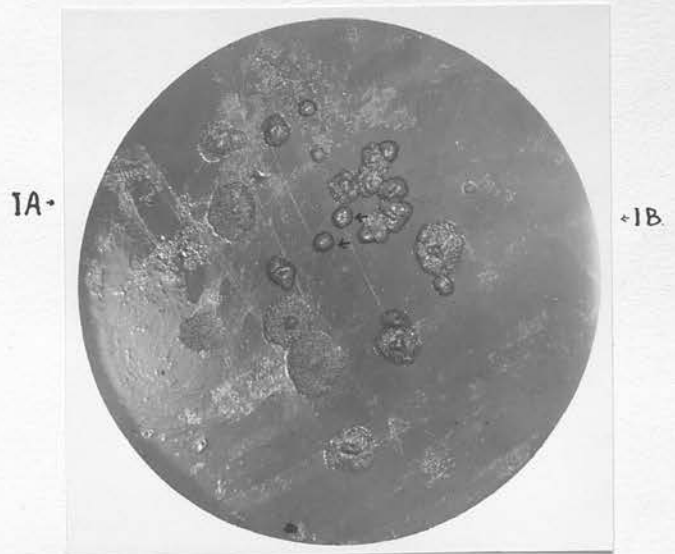


Fig. 8. Variants IA and IB.
73 days' growth.
Natural size.

Variant IB. One presented a glistening entire appearance but was not as smooth as Variant I and was sometimes characterized by a central cavity. Both types are represented in Fig. 8.

Variant IC. Three colonies developed which were identical in all respects with Variant I except for the fact that the periphery was broader than that possessed by even older colonies of the original variant.

When Variant IB was replated colonies similar to Variants I and II were observed and also a third type:

Variant IBi. This was a spherical, matt colony. It was more difficult to emulsify than Variant I and as it aged, frequently developed a thin, glistening, translucent, irregular periphery (Fig. 9).

Variant IC was replated with the following result:

Variant ICi. Only one colony appeared on these plates. It was composed of large, wax-like coils which were difficult to emulsify (Fig. 10).

Subcultures of Variant II were equally as unstable as those of Variant I. Several colonies similar to Variant I and many resembling Variant II developed. Others were the same as Variant IA.

Variant IIA. This type was characterized by a definite central cavity (Fig. 11).

Variant IIB. One variant was flat, glistening, translucent and irregular and resembled the periphery which developed on older colonies of Variant IBi.

A colony of Variant II was subcultured from the above/

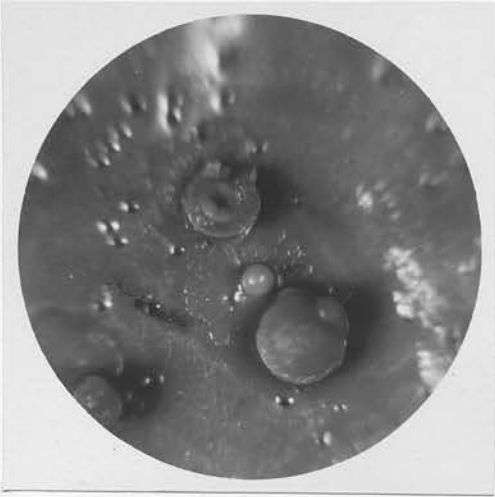
THE BOVINE TUBERCLE BACILLUS

Fig. 9. Variant IBi.
160 days' growth. x 10.



Fig. 10. Variant ICi.
120 days' growth. x 10.

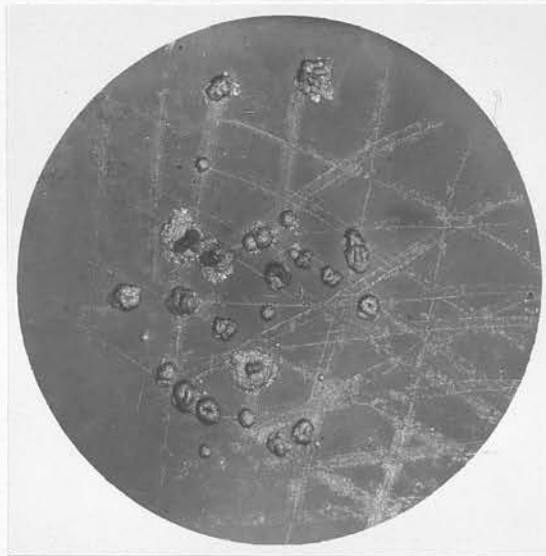


Fig. 11. Variant IIA. 58 days'
growth. Natural size.

above plates and several variants similar to the parent type developed.

Variant Ili. This form was large, round and glistening with a number of fine, short, straight lines giving a mosaic appearance to the smooth surface (Fig. 12). Both this variant and the following one gave a fairly even suspension in saline.

Variant Ii2. Rather similar to the above was a smaller, more spherical, glistening colony the surface of which was composed of lobes (Fig. 13).

Variant Ii3. A third variant was characterized by a central cavity from which radiated grooves dividing the colony into four or five segments. The surface was smooth and moderately glistening. The growth was not as emulsifiable as that of the two preceding colonies (Fig. 14).

Variant Ii4. A flat, irregular, rough type was also observed. It was difficult to emulsify (Fig. 15).

When Variant IIA was subcultured the plates developed colonies similar to Variant II, IBi and IIB. Variant IBi frequently formed large papillae.

When compared with the microphotographs and descriptions of S and R variants of the bovine tubercle bacillus (Petroff and Steenken, 1930) Variant I (Fig. 1 page 52) appeared to be practically identical with the S/

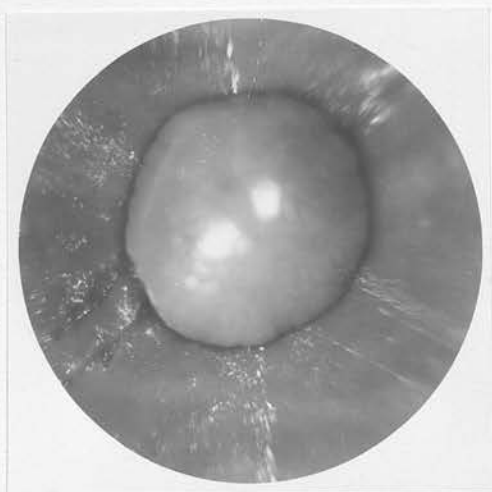
THE BOVINE TUBERCLE BACILLUS

Fig. 12. Variant IIIi.
63 days' growth. x 10.

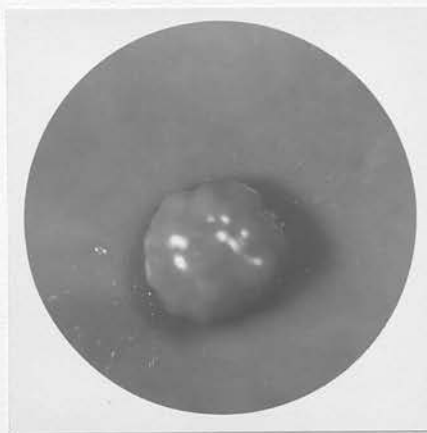


Fig. 13. Variant II2.
63 days' growth. x 10.

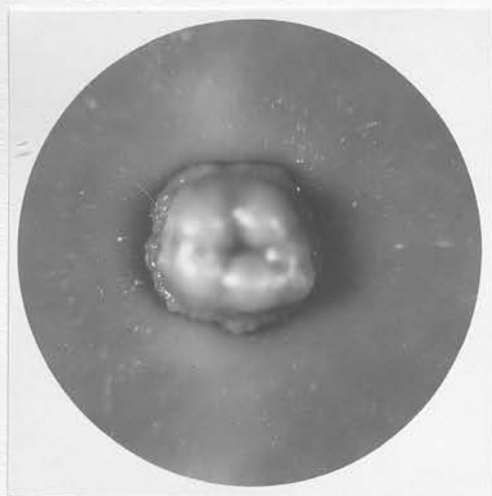


Fig. 14. Variant II3.
63 days' growth. x 10.

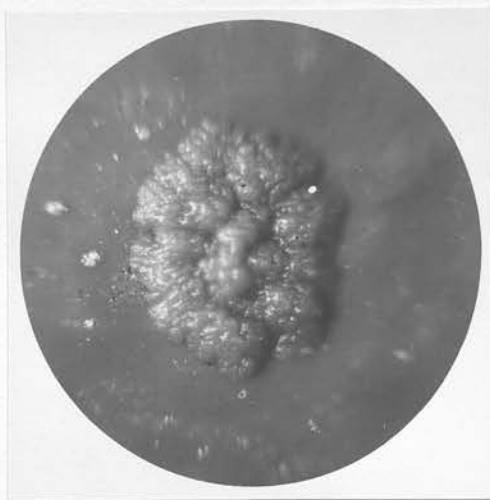


Fig. 15. Variant II4.
63 days' growth. x 10.

S form and Variant IA (Fig. 6 page 54) with the R.

In the case of the strain used for the experiments just recorded, there was no doubt that morphologically Variant I was an S form. There were, however, three variants, which, from the point of view of colony structure could be classified as R. These are illustrated in Figs. 4 (page 52), 10 (page 56) and 15 (page 58) (Variants II, ICi and II4 respectively). All were rough and difficult to emulsify and the bacilli of young colonies of Variants II and II4 were shorter than those of smooth colonies of the same age. Since approximately eight weeks growth was necessary before a colony acquired definite characters and since Variants ICi and II4 were only observed recently it has not been possible to test either their virulence or the permanency of their morphological characters.

It must be emphasized that while a number of different colony types have been described for each subculture there were also, in the majority of cases, other forms differing somewhat from those recorded. Judging from the limited number of subcultures made when studying this organism, it was evident that neither Variants I and II nor their descendants exhibited any marked degree of stability in subsequent generations.

Summary/

Summary of Observations.

The following table shows schematically how the different variants were derived.

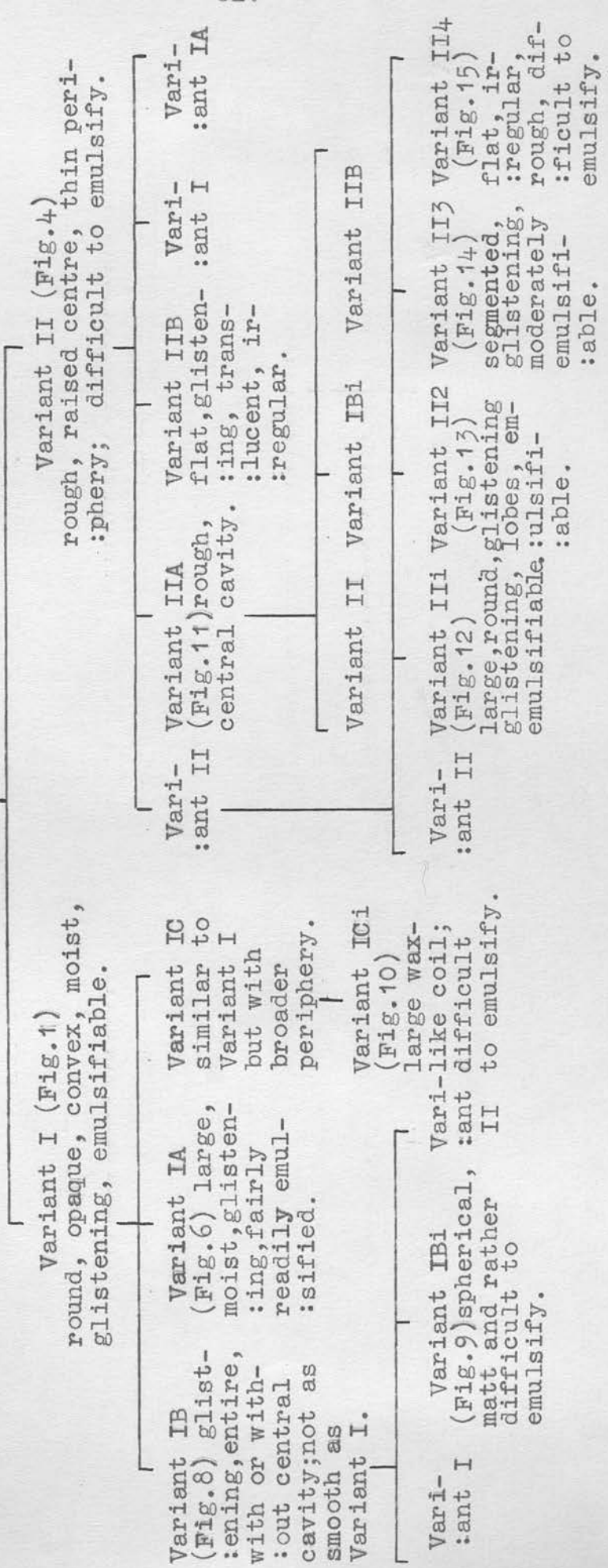
It may be seen that Variant II (Fig. 4, page 52 - a colony of rough structure) was derived from Variant I (Fig. 1, page 52 - a smooth colony) after two subcultures and that Variant I was derived from Variant II in the first subculture. In the second subculture of the latter type large, fairly smooth, glistening forms were observed as well as irregular rough colonies. Variant IA (Fig. 6, page 54) which closely resembled the R form described by Petroff occurred in the first subculture of Variants I and II and an extremely coarsely coiled colony (Variant ICi, Fig. 10, page 56) was obtained by plating Variant IC, a type closely resembling the smooth, glistening Variant I.

The results show that replating colonies of the strain of bovine tubercle bacillus used in these experiments may result in forms differing from the parent type, either slightly or in a very marked degree.

BOVINE/

BOVINE TUBERCLE BACILLUS

Undissociated Strain



Dissociation of a Recently Isolated Strain
of Tubercle Bacillus.

Another strain of tubercle bacillus was isolated from a guinea-pig which had been inoculated with the sediment from a milk sample. Moist, glistening colonies and finely furrowed variants were obtained from the first subculture on gentian-violet-egg plates. The glistening variant, when replated, formed smooth, convex, glistening colonies, but the growth was particularly slow and further subcultures were not made. It was evident, however, that dissociation occurred in a recently isolated strain as well as in one that had been cultivated artificially for a considerable period.

Pathogenicity Experiments.

In order to test their virulence colonies of Variants I, IA and II were emulsified in saline and injected intravenously into rabbits. Three animals were used for each variant and the amounts injected were 0.1 mgm, 0.01 mgm. and 0.005 mgm. respectively. Intradermal injections of a 1:1,000 dilution of Koch's Old Tuberculin were given at weekly intervals but in no instance did a definitely positive reaction result.

Variant I (Fig.1, page 52). (Three rabbits.) The two rabbits injected with 0.01 and 0.005 mgm. died of generalized tuberculosis in 133 and 136 days respectively. The former had extensive tuberculous lesions with demonstrable acid-fast organisms in the lungs, spleen and kidneys and in the enlarged axillary and left superficial inguinal glands. In the latter animal, acid-fast organisms were found in tuberculous lesions of the lungs, pericardium, spleen, kidneys and parietal peritoneum. The rabbit receiving a dose of 0.1 mgm. died in eight days from an intercurrent infection, acid-fast organisms being demonstrated in an enlarged cervical gland and in the spleen.

Variant/

Variant IA (Fig. 6, page 54). (Three rabbits.) The two rabbits receiving 0.1 and 0.005 mgm. of Variant IA died of generalized tuberculosis in 30 and 119 days respectively. In the former, extensive lesions with demonstrable acid-fast organisms were present in the lungs, liver, spleen, kidney and omentum and in enlarged cervical, mesenteric, superficial inguinal and lumbar glands. Post-mortem examination of the second rabbit revealed tuberculous lesions in the lungs, spleen, kidney and liver. Acid-fast organisms were found in all lesions, in the mediastinal glands and, in large numbers, in the spinal cord. The animal receiving 0.01 mgm. was killed after 143 days. Acid-fast organisms were demonstrated in lesions in the lungs, spleen and kidneys and in direct smears of the liver. The right axillary gland was slightly enlarged.

Variant II (Fig. 4, page 52). (Three rabbits.) The two rabbits receiving 0.1 mgm. and 0.01 mgm. were killed 143 days after injection. In the former, a few tubercles were found in the liver and spleen and numerous lesions containing acid-fast organisms were present in the lungs and kidneys. The left superficial inguinal and lumbar glands were enlarged. In the second animal there were a few tubercles in the lungs and histologic-
:al/

histological sections showed aggregations of lymphocytes in the liver. The rabbit receiving 0.005 mgm. died in 66 days from an intercurrent infection.

The virulence of Variants I, IA and II was also tested in guinea-pigs, six animals being injected with each variant, three receiving a dose of 0.1 mgm. and three 0.01 mgm. The injections were made in the left groin. Positive reactions to the intradermal tuberculin test usually occurred between three and eight weeks after inoculation.

Variant I (Fig.1, page 52).

Dose 0.1 mgm. (Three guinea-pigs.) The three guinea-pigs died in 45, 101 and 110 days respectively. In the first, the superficial inguinal glands on the left side were enlarged and caseous. Histological sections revealed tubercles in the spleen and liver and acid-fast organisms were demonstrated in a lesion at the site of injection, in the superficial inguinal glands and in the spleen. In the second animal acid-fast organisms were demonstrated in lesions in the liver, in the spleen, which was extremely enlarged, and in the superficial inguinal glands and local lesion. When examined histologically tubercles were found in the liver and spleen of the third guinea-pig and acid-fast/

acid-fast organisms were demonstrated in the left superficial inguinal gland which was enlarged and caseous.

Dose 0.01 mgm. (Three guinea-pigs.) These three animals died 14, 132 and 169 days after injection. In the former, tubercles were found in the spleen and acid-fast organisms were demonstrated in the spleen, the left inguinal gland, the superficial inguinal glands and lumbar glands and in a local lesion. In the second guinea-pig tubercles were found in the spleen and acid-fast organisms were demonstrated in the lungs. Post-mortem examination of the third animal revealed tubercles in the liver and spleen, acid-fast organisms being demonstrated in the latter organ as well as in the enlarged left superficial inguinal glands and local lesion.

Variant IA (Fig. 6, page 54).

Dose 0.1 mgm. (Three guinea-pigs.) One of the three animals died in 112 days. Tuberculous lesions were found in the lungs, liver and spleen and acid-fast organisms were demonstrated in the right and left superficial inguinal glands, in the lumbar glands and in a lesion at the site of injection. The second animal was killed after 187 days. Histological examination revealed a few aggregations of lymphoid cells in the lungs/

lungs and spleen. The inguinal and superficial inguinal glands were enlarged. The third animal died in ten days from an intercurrent infection.

Dose 0.01 mgm. (Three guinea-pigs.) Two of these died in 99 and 134 days respectively. In the former tuberculous lesions were found in the liver and in the spleen which was extremely enlarged. Acid-fast organisms were demonstrated in the superficial inguinal glands. In the second animal there were adhesions between the lungs and the chest wall and tubercles with associated acid-fast organisms were demonstrated in the lungs, liver and spleen. A lesion was present at the site of injection. The third animal was killed in 187 days. The inguinal, superficial inguinal and lumbar glands were enlarged and histological examination revealed tubercles in the spleen. Acid-fast organisms were demonstrated in the superficial inguinal glands.

Variant II (Fig. 4, page 52).

One guinea-pig injected with 0.1 mgm. died in 47 days. Examination of histological sections revealed aggregations of lymphoid cells in the lungs and tubercles in the spleen. Acid-fast organisms were demonstrated in a lesion at the site of injection. A second/

second guinea-pig injected with 0.01 mgm. was killed 187 days after injection and histological examination of the organs revealed tubercles in the spleen.

The remaining four animals died within seventeen days from an intercurrent infection.

Six more guinea-pigs were injected and the results were as follows:

Dose 0.1 mgm. (Three guinea-pigs.) One of the three died in 15 days, tuberculous lesions being demonstrated in the spleen. The second was killed in 53 days and when examined post-mortem slight enlargement of the superficial inguinal, lumbar and mediastinal glands was observed. Tubercles were found in the spleen and aggregations of lymphoid cells in the lungs. The third animal died in seven days from an intercurrent infection.

Dose 0.01 mgm. (Three guinea-pigs.) Two of the three animals died in 29 and 40 days respectively. Examination of histological sections revealed tubercles in the lungs, liver and spleen. There was slight enlargement of the superficial inguinal glands and in the animal which died after 40 days the lumbar glands were also enlarged. The third animal when killed after 53 days showed slight enlargement of the superficial inguinal gland and the mediastinal glands. There was a lesion at the site of injection. Histological examination revealed tubercles in the lungs and spleen.

Summary of
Pathogenicity Experiments with Rabbits.

Type of Variant injected.	Dose	Day of Death.	Organs in which tuberculous lesions were found post-mortem.
Variant I	0.01 mgn.	133rd	Lungs, spleen, kidneys, superficial inguinal glands.
Variant I	0.005 mgn.	136th	Lungs, pericardium, spleen, kidneys, parietal peritoneum.
Variant IA	0.1 mgn.	30th	Lungs, liver, spleen, kidney, omentum, all lymphatic glands.
Variant IA	0.01 mgn.	*143rd	Lungs, spleen, kidneys, axillary gland.
Variant IA	0.005 mgn.	119th	Lungs, spleen, liver, kidneys, spinal cord, mediastinal glands.
Variant II	0.1 mgn.	*143rd	Lungs, liver, kidneys, spleen, superficial inguinal, lumbar glands.
Variant II	0.01 mgn.	*143rd	Lungs, liver.
Variant II	0.005 mgn.	66th	No tuberculous lesions.

*killed.

Note:- Animals dying of intercurrent conditions not included in the table when death occurred within seventeen days of injection.

Summary of
Pathogenicity Experiments with Guinea-pigs.

Type of Variant injected.	Dose	Day of Death.	Organs in which tuberculous lesions were found post-mortem.
Variant I	0.1 mgm.	45th	Spleen, liver, superficial inguinal glands. (Local lesion)
Variant I	0.1 mgm.	101st	Liver, spleen, superficial inguinal glands. (Local lesion)
Variant I	0.1 mgm.	110th	Liver, spleen, superficial inguinal glands.
Variant I	0.01 mgm.	14th	Spleen, superficial inguinal glands. (Local lesion)
Variant I	0.01 mgm.	132nd	Spleen.
Variant I	0.01 mgm.	169th	Liver, spleen, superficial inguinal glands. (Local lesion)
Variant IA	0.1 mgm.	112th	Lungs, liver, spleen, superficial inguinal, inguinal, lumbar glands. (Local lesion)
Variant IA	0.1 mgm.	*187th	Spleen, lungs, inguinal, superficial inguinal glands.
Variant IA	0.01 mgm.	99th	Lungs, liver, spleen, superficial inguinal glands.
Variant IA	0.01 mgm.	134th	Lungs, liver, spleen. (Local lesion)
Variant IA	0.01 mgm.	*187th	Spleen, superficial inguinal glands.
Variant II	0.1 mgm.	47th	Spleen, lungs.
Variant II	0.1 mgm.	15th	Spleen.

Variant/

*killed.

Type of Variant injected.	Dose	Day of Death.	Organs in which tuberculous lesions were found post-mortem.
Variant II	0.1 mgm.	*53rd	Spleen, lungs, superficial inguinal, lumbar, mediastinal glands.
Variant II	0.01 mgm.	29th	Lungs, liver, spleen, superficial inguinal glands.
Variant II	0.01 mgm.	40th	Lungs, liver, spleen, superficial inguinal, lumbar glands.
Variant II	0.01 mgm.	*53rd	Lungs, spleen, superficial inguinal, mediastinal glands. (Local lesion)
Variant II	0.01 mgm.	187th	Spleen.

*killed.

Note:- Animals dying of intercurrent conditions not included in the table when death occurred within seventeen days of injection.

Though definite conclusions as to virulence could hardly be drawn from experiments in such small numbers of animals, it would appear that in rabbits the rough Variant II was of slightly lower virulence than the smooth Variant I and that Variant IA, which was similar to the relatively avirulent R form described by Petroff, was highly virulent. In guinea-pigs this type appeared to be slightly less virulent than Variant I.

Variant/

Variant II injected into rabbits and guinea-pigs appeared to be slightly less virulent than Variant I.

The small differences in the virulence of Variants were suggested by the survival time of the inoculated animals and to a less extent by the nature of the lesions found post-mortem.

The Attenuated Bovine Tubercle Bacillus
of Calmette and Guérin (B.C.G.).

Since Calmette and Guérin first advocated immunization against tuberculosis by means of the attenuated bovine tubercle bacillus now known as Bacillus Calmette-Guérin (B.C.G.), literature on the subject has rapidly accumulated. The majority of investigators support the view of Calmette that B.C.G. is avirulent not only for experimental animals but also for infants when administered according to the direction of the sponsors of this vaccine.

That B.C.G. can be dissociated into two types differing in their colony characters was first shown by Petroff, Branch and Steenken (1927), who separated variants corresponding in some respects to the R and S types observed in other bacterial groups.

Petroff and his co-workers also demonstrated a difference in virulence between these types analogous to that noted in the case of R and S variants of other organisms. The S type when injected by different routes resulted in generalized tuberculosis; the R type was practically avirulent. These authors pointed out, however, that, owing to the complexity in form of colonies of acid-fast bacilli "smooth" and "rough" were not/

not appropriate terms to apply to dissociates of this group of organisms. They suggested that "sensitive" and "resistant", the designations advocated by Hadley (1927), would be more suitable interpretations of the "S" and "R" when referring to colonies of B.C.G. In 1929 Petroff, Branch and Steenken showed that after injection with the undissociated or R type of growth progressive tuberculous disease, though rare, sometimes occurred. Two guinea-pigs out of eight died of generalized tuberculosis after injection of undissociated B.C.G. grown on glycerine-egg medium, while after injection with growth from a glycerine-bile-potato culture the death of only one animal out of 19 could be attributed to tuberculosis. In four of the latter examined between the 126th and 207th days the lymph nodes were enlarged and two of these also had tubercles in the spleen. In most instances the lesions were temporary and had a tendency to retrogress. As a possible explanation of the increase in virulence of the R type it was suggested that the development of antibodies in the injected animals effected the R \rightarrow S transformation such as occurs in vitro when an R variant is grown in contact with anti-R serum.

Further studies of dissociated strains of B.C.G. have/

have been made by a number of investigators. Kraus (1929) working with cultures dissociated by Petroff found the S strain to be virulent, but with cultures dissociated in his own laboratory he could find no difference in virulence among the variants and the undissociated strains. Petroff (1929) points out that the S colony dissociated by Kraus (1929) is not a true S but an "intermediate form". Among a large number of microphotographs of B.C.G. variants and their atypical forms Petroff and Steenken (1930) give an illustration of this "intermediate R" type which, owing to its round and smooth appearance has been regarded as an S variant by a number of investigators. Elbert and Gelberg (1930) inoculated guinea-pigs intraperitoneally with doses of 50 mgm. of the S, R and intermediate variants described by Petroff. They did not find the lesions produced in any way different from those resulting from a similar injection of undissociated B.C.G., i.e. local lesions which eventually healed and left no trace. Similar results were obtained by Nechtadimenko, Odrina, Syssak and Anguenitski (1930) who injected doses of 10, 50 and 100 mgm., by Piasecka-Zeyland (1929) and by Tzeknovitzer (1930). Begbie (1931) succeeded in dissociating B.C.G. into three types which he/

he named "S", "I" and "R", the "S" form being characterized by a central dome and flat spreading periphery. Of six guinea-pigs injected by the intracardiac route with subcultures from the S variant five died from systemic tuberculosis within forty days and the sixth when killed showed chronic tuberculous lesions in the internal organs. Of the six injected subcutaneously one showed miliary tubercles in the spleen, liver and lung; in another animal tuberculous lesions were found in the lungs; the remaining four animals had local lesions only. Six guinea-pigs were injected by the intracardiac route with subcultures from the R variant. Two died of generalized tuberculosis and two others showed chronic tubercular lesions in the spleen and lungs. When killed after sixty-seven days four of the six injected subcutaneously had systemic tuberculosis and two showed local lesions only. Injections of undissociated growth from Dorsett's egg medium exhibited more marked tubercular changes than those produced by subcultures from the I variant or from an undissociated strain of B.C.G. grown on glycerine-bile-potato.

B.C.G. in which dissociation has not purposely been induced, when cultivated on a medium other than glycerine-bile-potato has, in several instances, produced/

produced generalized tuberculosis or more extensive tubercular changes than those generally occurring after injection of growth from glycerine-bile-potato.

Uhlenhuth and Seiffert (1930) subcultured a strain of B.C.G. on Petroff's medium and injected the growth into guinea-pigs. One animal out of seven died of generalized tuberculosis. This strain was then re-injected into ten other guinea-pigs and all of them died of tuberculosis. Inoculation tests in rabbits proved it to be of the bovine type. Hutyra (1929) also produced generalized tuberculosis in guinea-pigs by injection of B.C.G. which had not been subcultured continuously on glycerine-bile-potato. Dreyer and Vollum (1931) working with deep bouillon cultures, after prolonged incubation, observed a granular type of growth at the foot of the flasks. Inoculation of guinea-pigs and rabbits with these granules resulted in a high mortality from tuberculosis and the strain was found to be virulent on subsequent inoculation into other animals. It corresponded in type-characters to neither the human nor the bovine varieties.

The extreme discrepancies of the results obtained in the study of B.C.G. and its dissociates indicated the need for further investigation of the subject.

Dissociation/

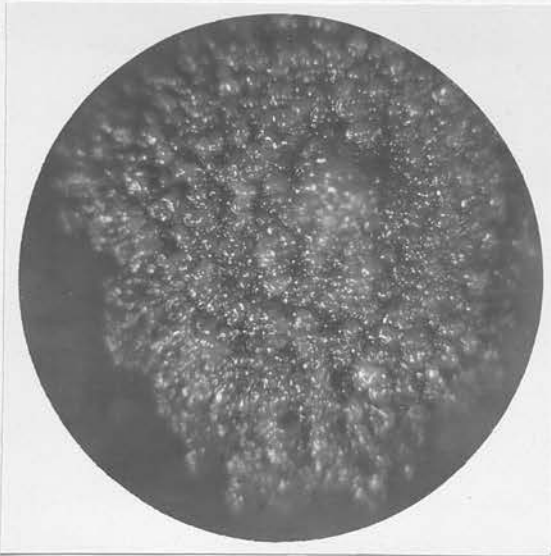
BACILLUS CALMETTE-GUÉRIN

Fig. 16. Variant I (S form) -
59 days' growth. x 10.



Fig. 17. Variant II (R form) -
59 days' growth. x 10.

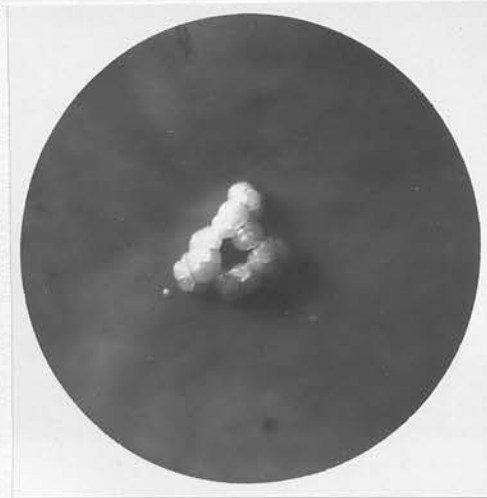


Fig. 18. Variant III (I form)
46 days' growth. x 10.

Dissociation Experiments.

A glycerine-bile-potato culture of B.C.G. (No.394) was obtained from the Pasteur Institute and was subcultured on glycerine-egg medium. Cultures were plated according to the technique of Petroff and Steenken (1930) as described on page 49, Petroff's medium with the addition of sodium taurocholate to differentiate colony types being used as before. The following three main types of colonies were observed on plates seeded from a glycerine-egg culture.

Variant I. This was a large spreading colony slightly raised in the centre and sloping gradually down to the edge. The entire surface was characterized by numerous small coils and furrows and the edge was thin and irregular. The growth was emulsifiable in physiological salt solution. This corresponds in most respects with the S type described by Petroff and will be referred to as the S variant in the text of this paper (Fig. 16).

Variant II. This colony consisted of large, white wax-like coils. The growth was broken up only with difficulty and did not form an even suspension. In every respect this colony corresponded with Petroff's R variant (Fig. 17).

Variant III. This was composed of wax-like coils surrounding a central cavity. It will be referred to as the I variant (Fig. 18).

The three types were subcultured on slopes of Petroff's medium and were used for pathogenicity experiments/

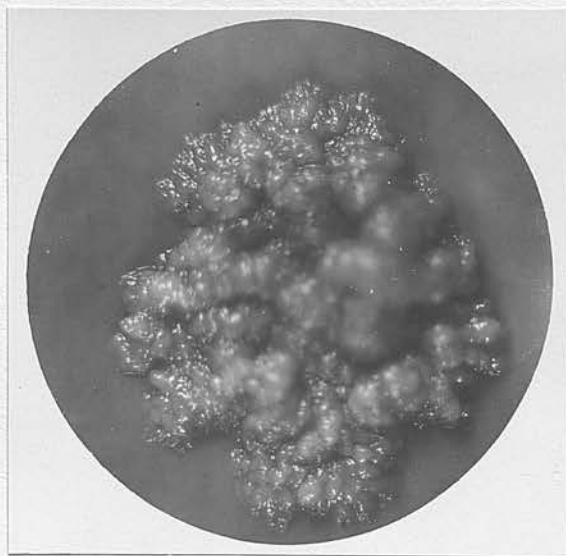
BACILLUS CALMETTE-GUÉRIN

Fig. 19. Atypical R form.
59 days' growth. x 10.

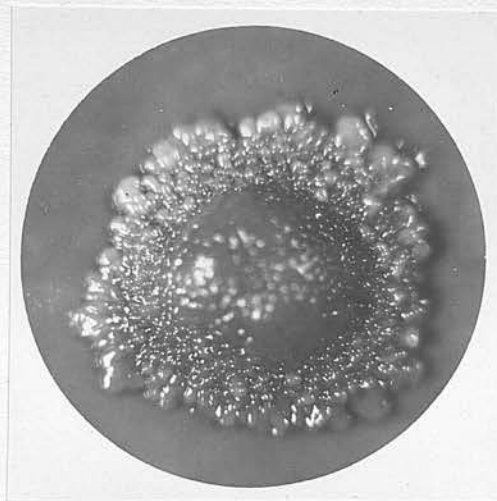


Fig. 20. S variant (described
by Begbie). 100 days' growth.
x 10.

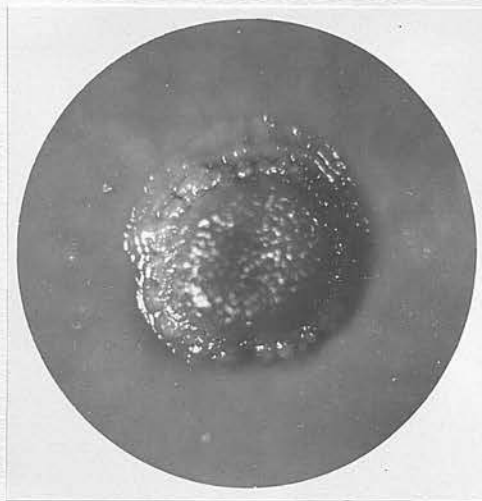


Fig. 21. I variant (described
by Begbie). 100 days' growth.
x 10.

experiments which will be described later.

Apparently variants of B.C.G. are not as constant in form as dissociates of many more rapidly growing organisms. Petroff, Branch and Steenken (1927) before inoculating animals subcultured their variants repeatedly until dissociation was well established. Kraus and Gerlach (1929), however, were unable to find any stability of form and their results were confirmed by Tzeknovitzer (1930).

Plates were inoculated periodically in order to determine whether or not the variants remained stable. Up to the present four subcultures in series have been made of the S variant and three of the R. A typical S colony was replated on fifteen plates. As a rule one or two S colonies were found but the majority differed from the original. One of the S variants was again replated with similar results, and so on. In no case have all the colonies of the subculture been identical with the type from which they were derived. After the fourth subculture of the S type only a few S colonies were found; the majority were R or atypical forms (Fig. 19). On this batch of plates were also observed the S (Fig. 20) and I (Fig. 21) variants described/

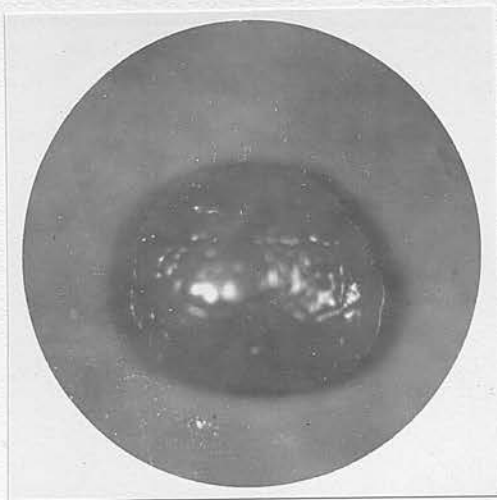
BACILLUS CALMETTE-GUÉRIN

Fig. 22. Variant described as S by some investigators. 108 days' growth. x 10.

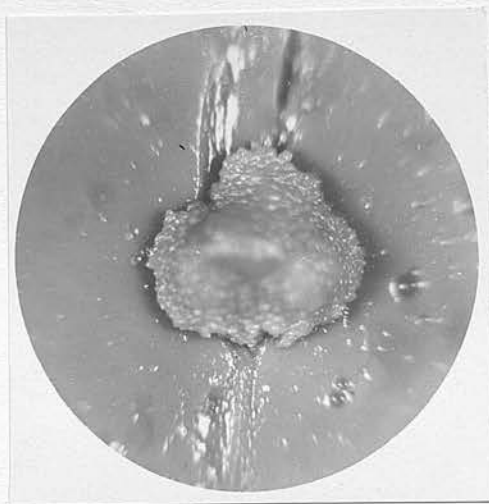


Fig. 23. Variant derived from replating S form (described by Begbie). 65 days' growth. x 10.

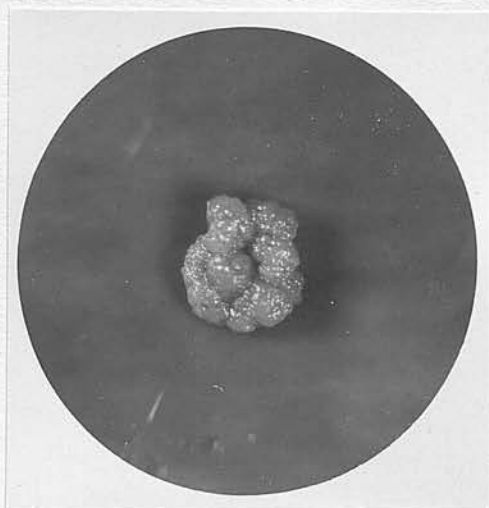


Fig. 24. Variant derived from replating S variant (described by Begbie). 65 days' growth. x 10.

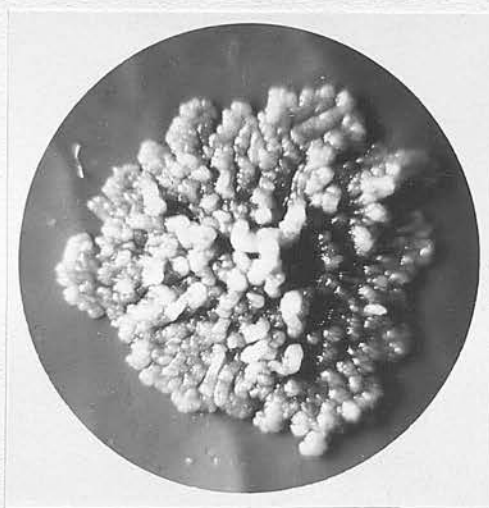


Fig. 25. Atypical S variant. 42 days' growth. x 10.

described by Begbie, and a round relatively smooth colony (Fig. 22) similar to the form which Kraus (1929) regarded as S but Petroff (1929) considered an "intermediate type". When replated the S form described by Begbie resulted in two different colonies, the one (Fig. 23) somewhat similar to the original in its structure and the ease with which it could be emulsified, the other (Fig. 24) more coarsely coiled and difficult to emulsify. A similar procedure of selection and plating was carried out using the R variant. In this case there was relative stability; for, although the resulting colonies might differ entirely from the parent colony they were still of a coarser structure than the S, the majority being characterized by large wax-like coils. In the three subcultures of the R variant S forms occurred only rarely. Frequently, however, a type occurred showing slight deviation from the S variant (Fig. 25). Some of the R forms are shown in Figs. 26, 27, 28 and 29, and one closely related form in Fig. 30.

On several occasions it was noticed that one type of R colony (Fig. 29) and also an atypical form (Fig. 31) developed a flat spreading border (Fig. 32) and the general nature of the growth closely resembled that of the/
the/

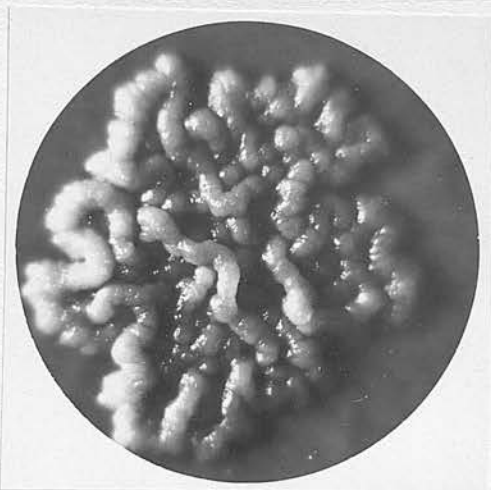
BACILLUS CALMETTE-GUÉRIN

Fig. 26. R variant. x 10.
46 days' growth.

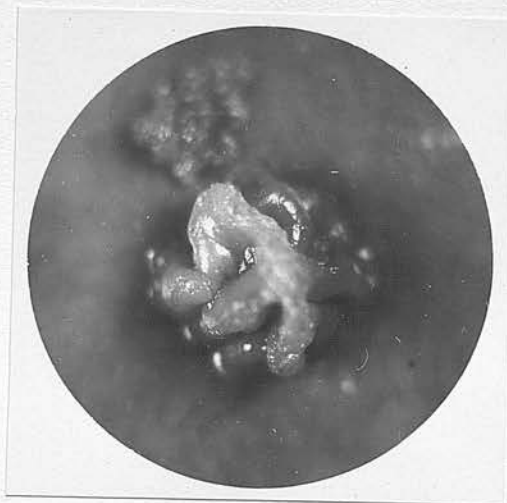


Fig. 27. R variant. x 10.
59 days' growth.

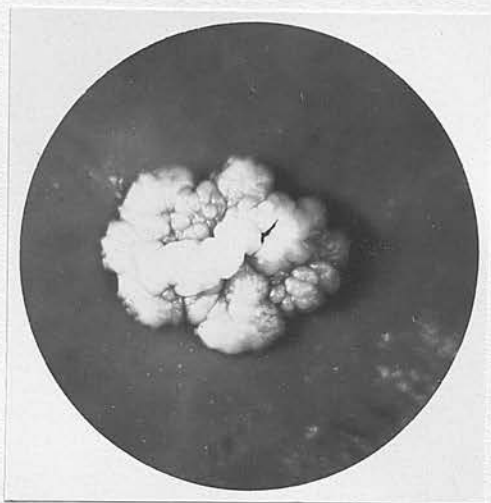


Fig. 28. R variant. x 10.
70 days' growth.

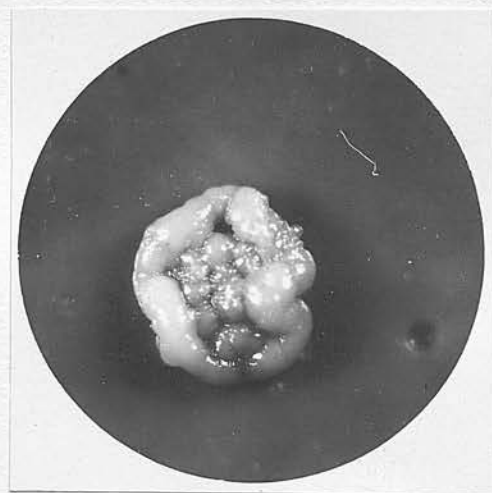


Fig. 29. R variant. x 10.
59 days' growth.

BACILLUS CALMETTE-GUÉRIN

Fig. 30. Atypical R variant.
46 days' growth. x 10.

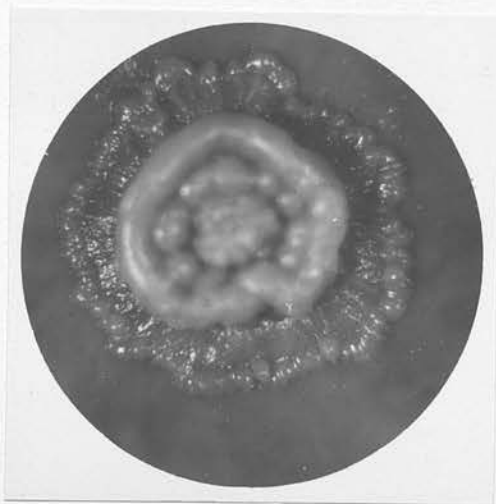


Fig. 31. Atypical R variant
with flat border. 136 days'
growth. x 10.

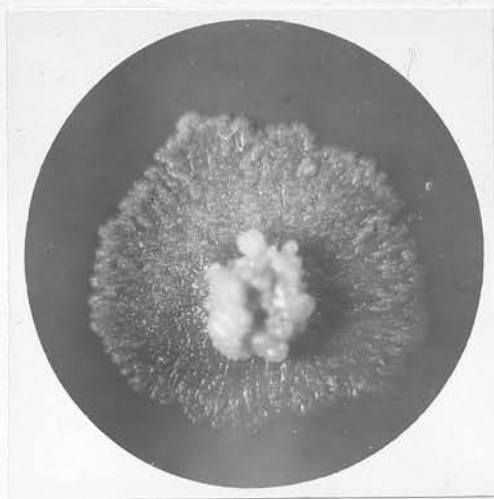


Fig. 32. R variant with flat,
spreading border. 84 days'
growth. x 10.

the S variant. When it was replated, however, colonies of a coarsely coiled structure resulted (Fig. 33), and a subculture of this type gave colonies of a somewhat similar appearance (Fig. 34). Later the flat border and the original colony became covered with a mass of smooth, glistening papillae (Fig. 35) some of which enlarged till they formed distinct "super-growths". From this another flat wrinkled border grew and this again became covered with papillae. The young smooth papillae could be removed in their entirety when touched with the end of an inoculating wire. When replated, colonies of the R type resulted (Fig. 36), and all were of the same form. Papilla formation was not unusual on other colonies of B.C.G. It was observed on S and atypical forms (Fig. 37), on the S variant described by Begbie (Fig. 38) and after prolonged incubation on the outer coils of R variants.

This illustrates the extreme complexity of morphology and morphological variation displayed by cultures of the organism studied.

In order to determine whether variation occurred in vivo two guinea-pigs were each inoculated subcutaneously with the entire growth from a glycerine-bile potato culture of B.C.G. which had not previously been subcultured/

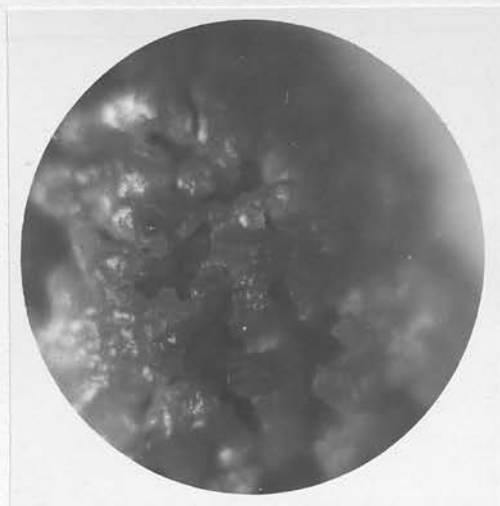
BACILLUS CALMETTE-GUÉRIN

Fig. 33. Variant resulting from plating spreading border shown in Fig. 32. 77 days' growth. x 10.

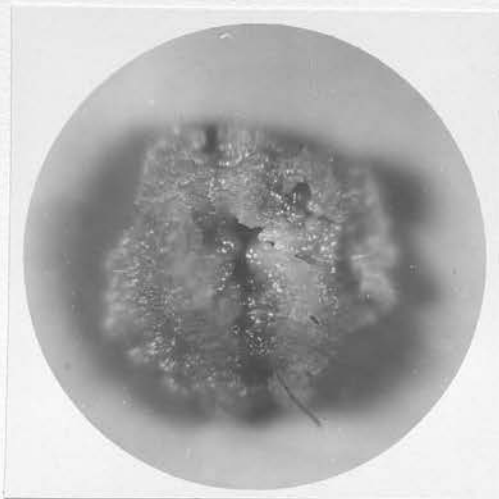


Fig. 34. Variant resulting from plating colony shown in Fig. 33. 89 days' growth. x 10.

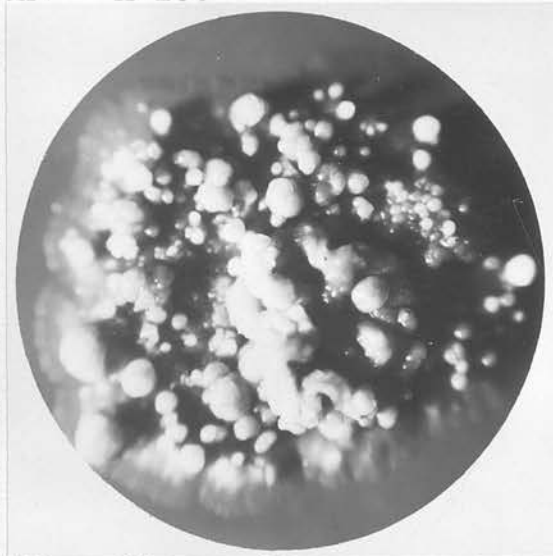


Fig. 35. Papillae covering R variant and flat border. 129 days' growth. x 5.

BACILLUS CALMETTE-GUÉRIN

Fig. 36. Variant resulting from replating papilla. 59 days' growth. x 10.

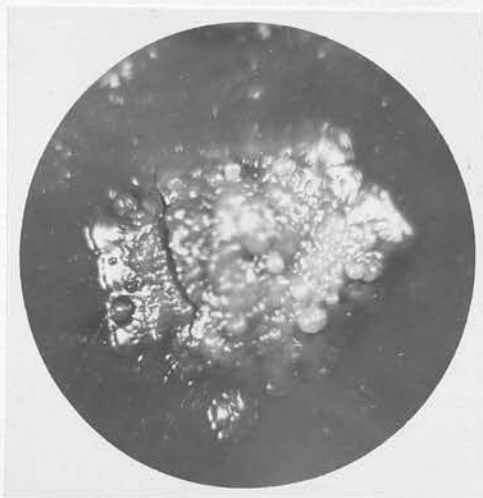


Fig. 37. Atypical S variant showing papillae. 110 days' growth. x 10.

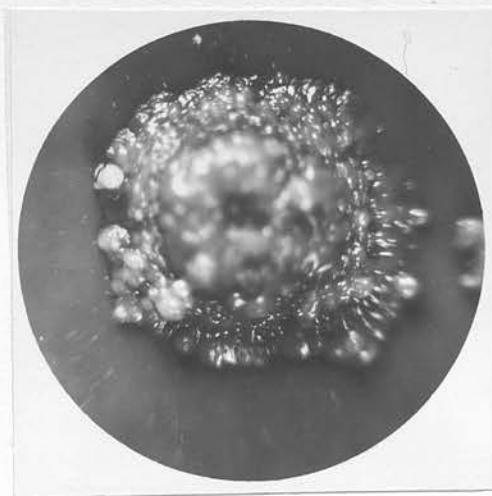


Fig. 38. S variant (described by Begbie) showing papillae. 123 days' growth. x 10.

subcultured on any other medium. After twenty days pus was removed and inoculated directly on gentian-violet-egg plates. Only one colony developed.

(Plates inoculated with pus treated with antiformin or filtered and plated with and without further dilution showed no growth.) The colony was large, flat, spreading, slightly glistening and furrowed and had a fairly regular edge. It was unlike any of the forms resulting from inoculation of growth from a glycerin-bile-potato culture on gentian-violet-egg medium or any of the types observed in subcultures of the different variants. When replated a similar form was obtained. Apparently the colony was avirulent since the two guinea-pigs survived for four months and although showing a positive tuberculin reaction after this time, when killed and examined post-mortem they revealed no macroscopic evidence of tuberculosis.

Attempts were made to obtain individual colonies from the lesions of animals injected with typical S and R variants but either no growth resulted or the plates became contaminated before the colonies were sufficiently developed to be used for comparison.

Pathogenicity/

Pathogenicity Experiments.

Guinea-pigs were used for these experiments.

In every instance except one the dose of culture injected was 5 mgm. moist weight in 0.5 c.c. of saline. Injections were in certain tests intracardiac and in others subcutaneous (in the left thigh). Animals which survived were killed and examined at intervals varying from 41 days to 241 days. In all cases an autopsy was performed. Attempts were made to demonstrate acid-fast bacilli in internal organs and lesions. Histological examination of lungs, liver, spleen and kidney (and where necessary of brain and glands) was carried out in the majority of cases.

Before commencing tests with dissociated strains six guinea-pigs were each injected with 10 mgm. (moist weight) of growth from a glycerine-bile-potato culture received from the Pasteur Institute. Five of the animals when killed after three months showed no sign of tuberculosis. Tuberculin tests made two days before the animals were killed yielded in three cases faintly positive reactions; in the other two animals the reaction was negative. (One animal died in five days from an intercurrent infection.)

With a view to ascertaining whether B.C.G. when grown/

grown on a medium other than glycerine-bile-potato might revert to a virulent state and cause progressive or generalized tuberculosis, subcultures were made from glycerine-bile-potato on glycerine-egg and were maintained on this medium through six successive transfers. Six guinea-pigs were each injected with 5 mgm. The results are as follows:-

Subculture of Undissociated B.C.G. on Glycerine-Egg Medium.

Intracardiac injection: Three animals were inoculated and examined after 56, 190 and 241 days respectively. They were all free from tuberculous lesions.

Subcutaneous injection: Three animals were inoculated and examined after 190, 193 and 241 days respectively. In one a tuberculous lesion containing acid-fast bacilli was present at the site of injection. In the second animal no lesions were found but microscopic examination of internal organs revealed acid-fast bacilli in the testis. The third animal showed no evidence of tuberculosis.

Subculture of Dissociated B.C.G.

Subcultures were made from the S,R and I colonies and the resulting growths were injected into guinea-pigs each receiving a dose of 5 mgm. Tuberculin tests were made at intervals of one week; a positive reaction/

reaction appeared usually between the third and seventh weeks. The results of the experiments were as follows:-

Subculture of S variant.

Intracardiac injection (Six guinea-pigs): Two animals died in 40 and 142 days respectively with tuberculous lesions of the pericardium. One also showed caseous tubercles in the heart wall and in the intestine and the other tuberculous lesions in the parietal and visceral pleura and a few small tubercles in the lungs; in the latter the mediastinal glands were enlarged and caseous. It would seem that injection by the intracardiac route results in the deposition of a certain number of organisms in the pericardium or heart wall and causes a local cardiac or pericardiac lesion. The third animal when killed after 146 days showed one or two small subcapsular lesions in the kidneys with demonstrable acid-fast organisms. The other three animals showed no evidence of tuberculosis.

Subcutaneous injection (Six guinea-pigs): Local lesions were found in two animals one of which died of an intercurrent infection after 54 days and the other when killed in 146 days. A third animal examined after 128 days showed no macroscopic evidence of tuberculosis/

tuberculosis but after prolonged search a few acid-fast organisms were found in smears from one testis and from bone marrow. Three other animals examined after 41, 80 and 128 days respectively were found free from tuberculous lesions.

Subculture of R variant.

Intracardiac injection (Six guinea-pigs): Of four animals killed after 146 days only one showed evidence of tuberculous infection. This animal had a caseous substernal lesion and large tubercles containing acid-fast organisms in the pericardium. One animal which died in 17 days of an intercurrent condition and a second which died in 48 days of acute intestinal obstruction showed no sign of tuberculosis.

Subcutaneous injection (Six guinea-pigs): Four animals were examined after 146 days only one showing a small tuberculous lesion which was localized at the site of injection. Two died of intercurrent conditions on the 34th and 99th days respectively, the former having a tuberculous lesion at the site of injection.

Subculture of I variant.

Intracardiac injection (Five guinea-pigs): One animal died in six days. Sections of brain showed commencing miliary tubercles in the cortex containing clumps of acid/

acid-fast bacilli. The organisms were also demonstrable in the lungs, liver and spleen, but were not associated with lesions. Two animals examined after 70 and 110 days and two after 155 days showed no evidence of tuberculosis.

Subcutaneous injection (Six guinea-pigs): No evidence of tuberculosis was found in these animals, except a local lesion in one which died after 12 days. The remaining five were killed and examined after 155 days.

To ascertain whether there was any difference in virulence between S variants injected directly from plates and subcultures of this type, a number of S colonies were removed from the plates, emulsified and injected, each animal receiving a dose equivalent to 5 mgm. of moist growth.

Inoculation with S variant.

Intracardiac injection (Three guinea-pigs): One animal died in 21 days. Caseous tuberculous lesions were present in the spleen and section showed early tubercles in the liver. Acid-fast organisms without detectable lesions were also demonstrated in the lungs. A second animal when killed after 133 days showed a large caseous tuberculous lesion in the pericardium and several smaller ones associated with adhesions between heart and diaphragm/

diaphragm. A caseous lesion was present in the pleura. Acid-fast organisms were demonstrated in these. (One of the animals inoculated died in 8 days apparently as a result of a parasitic infestation.)

Subcutaneous injection (Three guinea-pigs): The three animals in this group were killed after 133 days. Two showed tuberculous lesions at the site of injection; the third was quite free from tuberculous disease.

To determine whether any increase in virulence had occurred during further subcultivation on gentian-violet-egg medium S and R colonies were again emulsified and injected into guinea-pigs, each animal receiving approximately 5 mgm. of moist growth.

Inoculation with S variant.

Intracardiac injection (Two guinea-pigs): One animal died in 94 days showing tuberculous lesions associated with adhesions between heart and diaphragm and one small lesion in the visceral pleura. A tuberculous lesion on the diaphragm and another in the visceral pleura were found in the second animal when killed in 119 days. Acid-fast organisms were demonstrated in all lesions.

Subcutaneous injection (Two guinea-pigs): When killed after/

after 119 days in one animal a large lesion was found at the site of injection; the other showed no evidence of tuberculosis.

Inoculation with R variant.

Intracardiac injection (Two guinea-pigs): One animal when killed after 119 days showed several tuberculous lesions in the visceral pleura and adhesions between the lungs and chest wall. (The second animal died three days after injection; the only pathological condition noted was fatty infiltration of the heart, liver and kidneys.)

Subcutaneous injection (Two guinea-pigs): There was no evidence of tuberculosis in either of the two animals when killed after 119 days.

To ascertain the virulence of the S variant corresponding to that described by Begbie, several colonies of this type were emulsified and injected into six guinea-pigs, each animal receiving a dose of 5 mgm.

Intracardiac (Three guinea-pigs): The three animals were killed in 96 days. Acid-fast organisms without detectable lesions were demonstrated in the liver of one; the others showed no evidence of tuberculous infection.

Subcutaneous/

Subcutaneous injection (Three guinea-pigs): When killed after 96 days acid-fast organisms without detectable lesions were demonstrated in the lungs of one animal. The other two were apparently free from tuberculous infection.

The results of the pathogenicity experiments are summarized in the following tables.

Summary of Pathogenicity Experiments with B.C.G.Animals Injected

Intracardiac					
	Tuber- :culous lesions in heart wall, peri- :cardium and inter- :nal org- :ans.	Tuber- :culous lesions in peri- :cardium and heart wall on- :ly.	Tuber- :culous lesions in in- :ternal organs.	No tuber- :culous lesions.	No of ani- :mals.
Undissociated B.C.G. on gly- :cerine-bile- potato.	-	-	-	2	2
Undissociated B.C.G. on gly- :cerine-egg- medium.	-	-	-	3	3
Subculture of R variant.	-	1	-	4	5
Subculture of I variant.	-	-	1	4	5
Subculture of S variant.	2	-	1	3	6
S colonies.	1	-	1	-	2
S colonies (af- :ter further sub- :cultivation on gentian-violet- egg medium).	-	-	2	-	2
R colonies (af- :ter further sub- :cultivation on gentian-violet- egg medium).	-	-	1	-	1
S colonies (as de- :scribed by Begbie)	-	-	-	3	3
Total	3	1	6	19	29

Note: Animals dying of intercurrent conditions not included in the table when death occurred within three weeks of injection.

Summary of Pathogenicity Experiments with B.C.G.Animals Injected

	Subcutaneous			No. of animals.
	Local tuberculous lesions.	Tuberculous lesions in internal organs.	No tuberculous lesions.	
Undissociated B.C.G. on glycerine-potato.	-	-	3	3
Undissociated B.C.G. on glycerine-egg-medium.	1	-	2	3
Subculture of R variant.	2	-	4	6
Subculture of I variant.	-	-	5	5
Subculture of S variant.	2	-	3	5
S colonies	2	-	1	3
S colonies (after further subcultivation on gentian-violet-egg medium).	1	-	1	2
R colonies (after further subcultivation on gentian-violet-egg medium).	-	-	2	2
S colonies (as described by Begbie)	-	-	3	3
Total	8	-	24	32

Note: Animals dying of intercurrent conditions not included in the table when death occurred within three weeks of injection.

Discussion.

The observations recorded form a contribution to the study of Calmette and Guérin's attenuated tubercle bacillus (B.C.G.) and its dissociates. The experiments fail to establish type-stability among the descendants of the various colony forms when subcultured on gentian-violet-egg medium and the results are contrary to those of Petroff, Branch and Steenken (1927, 1929) who first recognised the S and R types of B.C.G. and who claimed that they were relatively stable. On the other hand, the results correspond in certain respects to those of certain other investigators (Kraus and Gerlach, 1929; Tzeknovitzer, 1930) who found the various types to be reversible.

S, R and I variants and the S form described by Begbie as well as atypical forms of B.C.G. were observed. The S colony was spreading and characterized by a finely coiled and furrowed surface and irregular edge. It was emulsifiable in normal saline; and when replated proved to be particularly unstable. The R colony was composed of large white wax-like coils. A number of colonies which differed from each other in actual form but in virtue of their coarsely coiled structure and the difficulty with which they were emulsified/

emulsified in normal saline, were included in the group of R variants. When these were subcultured the resulting colonies rarely resembled the type from which they were derived. The majority exhibited the coarsely coiled R structure but occasionally atypical S or true S forms (according to Petroff's classification) occurred. The I variant was characterized by wax-like coils surrounding a central cavity.

Subcultures on slopes of Petroff's medium were made from the various colonies and the growth was injected into guinea-pigs before subsequent plating revealed the fact that the types were unstable. It is probable therefore that a change in type occurred on the slopes prior to inoculation. After injection with B.C.G., either dissociated or undissociated, lesions usually remained localised at the site of injection, in some cases disappearing within a short time and in others persisting until the animals were killed at intervals of from four to six months later. Lesions which occurred in the pericardium or heart wall in four animals, were probably due to the deposition of a certain number of organisms during injection by the intracardiac route. Undissociated B.C.G. on glycerine-bile-potato was avirulent when injected into guinea-pigs. When grown on glycerine-egg medium and after subculturing/

subculturing on Petroff's medium from R and I variants it was still relatively avirulent. After injection with growth from subcultures of the S variant three of eleven animals exhibited systemic tuberculosis, two of these also having tuberculous lesions in the pericardium. Similarly, after injection of S colonies direct from plates two animals of six inoculated showed tubercles in the internal organs. After further subcultivation on gentian-violet-egg medium two animals of four showed tuberculous lesions following injection with the S variant and one animal of three on injection with the R variant.

Petroff stresses the close relationship between virulence and the S type and although Kraus has confirmed this working with variants received from Petroff he and various other investigators have failed to confirm it with cultures dissociated in their own laboratories. Begbie, moreover, has shown that an even higher degree of virulence than that obtained by Petroff in his experiments resulted after injection with growth from subcultures of a colony similar to, though not identical with, the S variant. This colony is described as consisting of a raised central dome and a spreading fringe or margin. The surface of the/

the central dome was smooth and the surface of the fringe characterised by numerous small round elevations. According to Petroff's theories a possible explanation for the marked virulence associated with this colony would be that the border was composed either of S or atypical S growth and when subcultured prior to inoculation a large number of S forms developed, and these when injected proved to be highly virulent. Dissociation after subculturing from the R variant might also account for the cases of generalized tuberculosis occurring in that group. The results recorded in this paper fail to confirm those of Begbie (1931). None of the six animals injected with colonies identical with the S form which he described showed evidence of tuberculosis when killed after 96 days. This shows that a particular type of variant dissociated from one culture at a different time may be completely lacking in virulence. It seems evident therefore that variation in virulence like many other changes which are now recognized features of dissociation may occur unexpectedly and for no reason which can be explained in the present state of our knowledge.

Undoubtedly the individual resistance of the respective animals plays an important part, especially where/

where the infecting organism is only of a mildly virulent nature. This in itself, however, cannot be considered a sufficient explanation for such widely divergent results. Actual differences in the strains used by various investigators are probably of equal importance. Cultures of B.C.G. used for experimental purposes have frequently been in the hands of investigators for some time and even although cultivated on glycerine-bile-potato it is possible that slight differences in technique employed in the making of the medium or differences in the batches of potatoes or bile may play a more important part in causing variation among strains than is realized at present.

It seems somewhat significant that in the majority of instances in which progressive tuberculosis or extremely severe tuberculous changes have occurred in experimental animals the infecting strain of B.C.G. has at some time or other been cultivated in a manner other than that advocated by Calmette for the preparation of the vaccine, but it is evident from the reports of many investigators who have introduced B.C.G. into experimental animals and infants that the vaccine when prepared and administered strictly according to the direction of Calmette is apparently avirulent. While in the/

the experiments recorded in this paper, most of the data indicate the avirulence of the organism, the fact remains that when cultures from an S dissociate were inoculated into guinea-pigs in seven cases the results showed that this derivative possessed a certain degree of virulence though of course in no way comparable to that of a recently isolated bovine tubercle bacillus. The observations of certain workers have suggested that B.C.G. does not possess a fixed or stable avirulence and that it may yield, under experimental conditions, a virulent derivative. Apart from pathogenicity the observations recorded reveal a remarkable variability in the biological characters of the organism and the possibility of its regaining virulence under conditions in which it is used for practical immunisation must remain an open question.

THE HUMAN TUBERCLE BACILLUS

Two variants of the human tubercle bacillus have been described by Petroff and Steenken (1930). One form, probably an R, was waxy and raised, with large folds, and became yellow with ageing. The S type was flat, spreading, composed of small wrinkles, and, unlike the R form, was easily emulsified in saline. Other colonies were also observed but a description of these and the results of the pathogenicity experiments have not yet been published.

Dissociation Experiments.

A strain of human tubercle bacillus which had been kept in the laboratory and subcultured periodically on glycerine-egg medium, was used for the following experiments.

Two variants appeared on these plates, the one large and spreading, the other smaller, much raised and the surface like closely packed papillae. Both were subcultured on slopes of gentian violet egg medium before being replated.

When growth from the subculture of the large, spreading colony was emulsified and plated the following/

THE HUMAN TUBERCLE BACILLUS

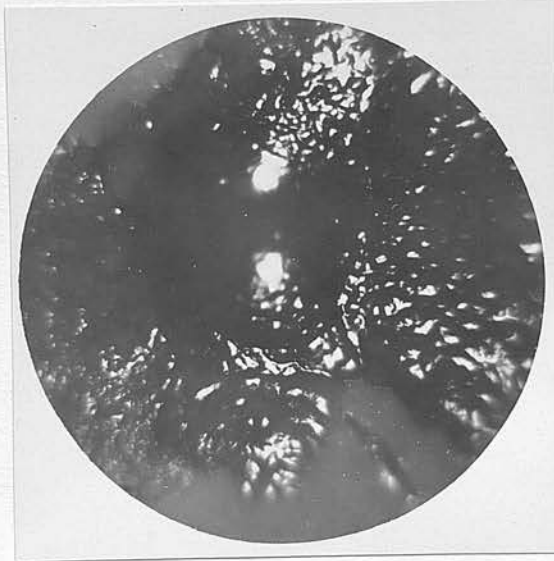


Fig. 39. Variant IA.
143 days' growth. x 10.

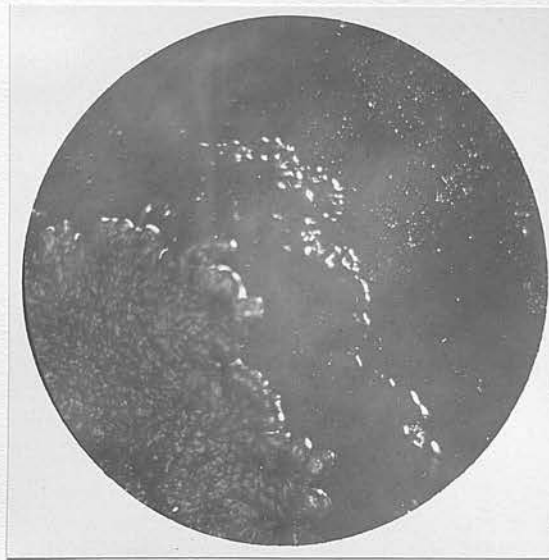


Fig. 40. Variant IB.
143 days' growth. x 10.

following type of colony was observed:-

Variant IA. This was a large, convex, glistening colony with a slightly undulating surface. As the colony aged a thick, glistening, irregular border developed (Fig. 39).

Variant IB. On the same batch of plates was a flat, matt, irregular colony which threw off a thick, glistening border (Fig. 40).

When replated the convex, glistening colony described as Variant IA resulted in three types of colonies.

Variant IAi. This was a large, matt colony possessing a granular centre and a spreading, finely wrinkled periphery. It was white in colour and relatively easy to emulsify (Fig. 41).

Variant II. The structure of this colony was similar to the small variant observed when the organism was plated for the first time. It was moist, glistening, beaded and extremely raised, giving a conical effect. It was of a reddish brown colour, difficult to emulsify in saline and tended to become slightly flattened as it aged. (Fig. 42).

Variant IA2. The third variant on these plates combined the characters of both the types just described. It had a moist, raised centre and a flat, matt, finely wrinkled periphery (Fig. 43).

When growth from the subculture of the original small, raised colony similar to Variant II was replated the following types of colonies were observed:

Variant IAi. This was similar to Variant IAi described above and as it aged it developed secondary areas of growth at the edge (Fig. 44).

Variant/

THE HUMAN TUBERCLE BACILLUS

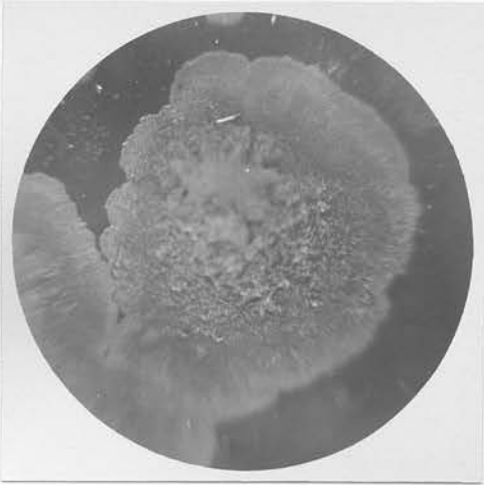


Fig. 41. Variant IAi.
82 days' growth. x 10.

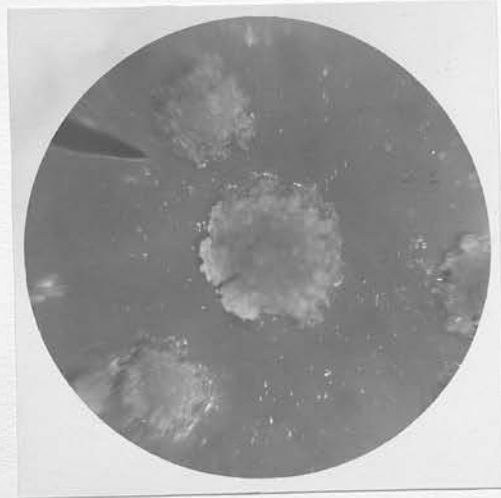


Fig. 42. Variant II.
82 days' growth. x 10.

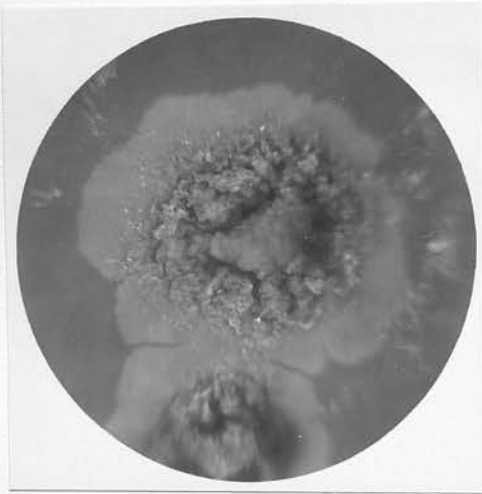


Fig. 43. Variant IA2.
82 days' growth. x 10.

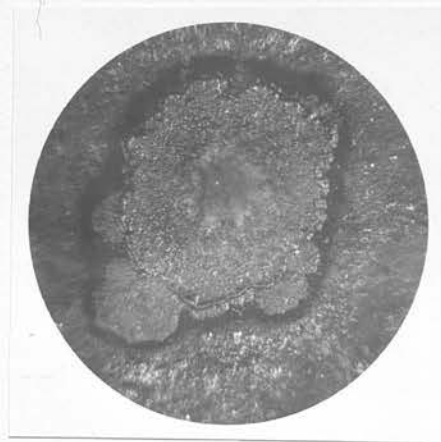


Fig. 44. Variant IAi.
154 days' growth. x 10.

THE HUMAN TUBERCLE BACILLUS

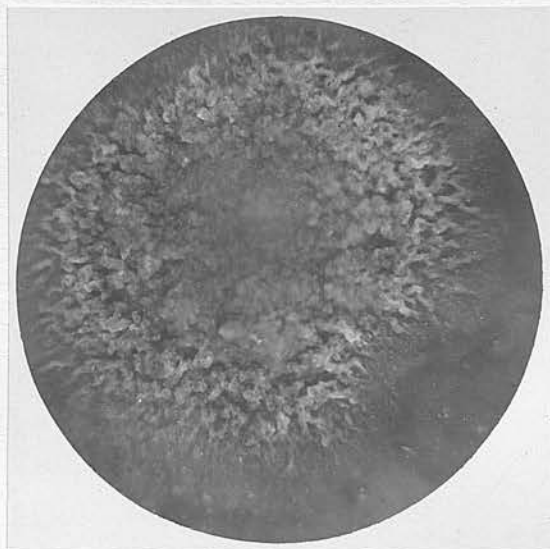


Fig. 45. Variant IIA.
154 days' growth. x 10.

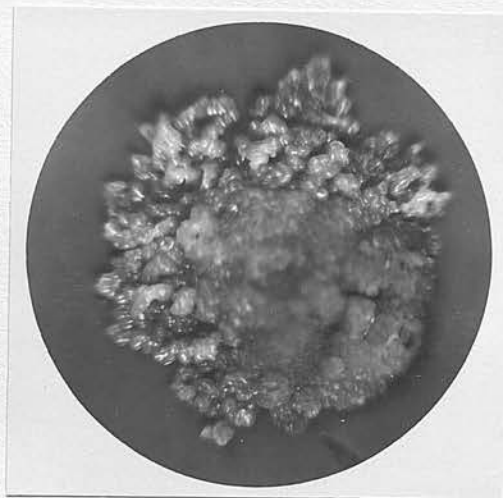


Fig. 46. Variant IIIi.
65 days' growth. x 10.

Variant IIA. The wrinkles in this type were very much coarser in structure than those of Variant II and the centre of the colony was brown (Fig. 45).

When Variant IAi from this batch of plates was subcultured the resulting colonies did not resemble the parent type:

Variant III. The centre of this variant is uneven and more raised than the periphery which is composed of irregular coils (Fig. 46).

Variant II2. Unlike the parent type this colony is cream coloured and presents a beaded appearance (Fig. 49). The colonies represented in Figs. 47 and 48 were also observed on these plates and may be stages in the growth of Variant II2.

Summary of Observations.

The following shows schematically how the different variants were derived:-

Human/

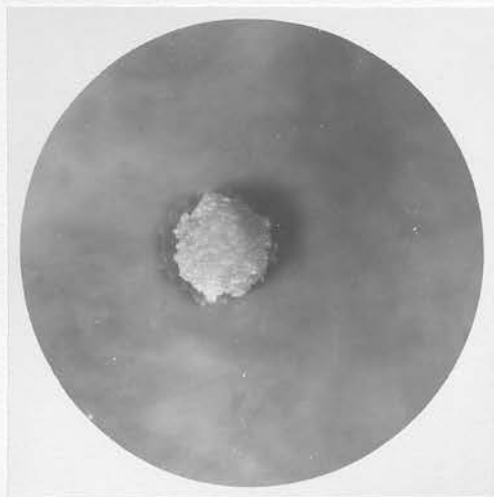
THE HUMAN TUBERCLE BACILLUS

Fig. 47. 65 days' growth.
x 10.

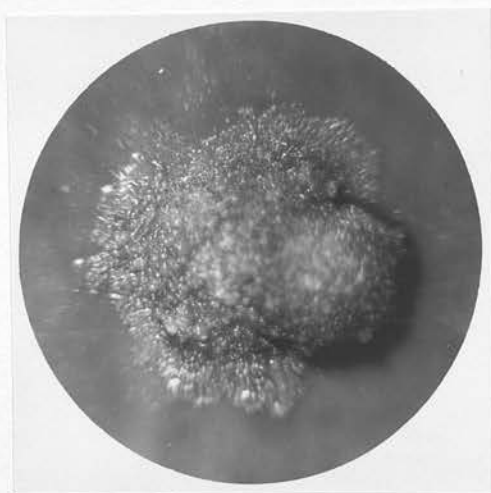


Fig. 48. 65 days' growth.
x 10.

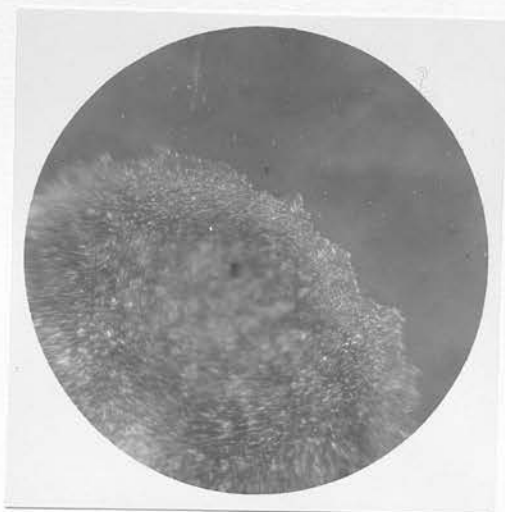


Fig. 49. Variant II2.
65 days' growth. x 10.

HUMAN TUBERCLE BACILLUS

Undissociated Strain

Variant I
large, spreading, beaded.

Subculture on gentian-violet-egg slope.

Variant IA (Fig. 39)
large, convex, glistening,
undulating.

Variant IB (Fig. 40)
flat, matt, irregular,
glistening periphery.

Variant IAi (Fig. 41)
large, matt, granular
centre, finely
wrinkled periphery,
relatively easily
emulsified.

Variant IA2 (Fig. 43)
moist, raised centre,
finely wrinkled, matt
periphery, difficult
to emulsify.

Variant II

Variant II (Fig. 42)
small, moist, glistening, conical,
beaded, difficult to emulsify.

Subculture on gentian-violet-egg slope.

Variant IIA (Fig. 45)
large, spreading, coarse,
wrinkled.

Variant IAi

Variant IA Variant Iii (Fig. 46) Variant II2
raised, irregular, (Fig. 49)
rough, difficult large, spread-
to emulsify. :ing, beaded.

From the foregoing table it may be seen that although replated three times the descendants of Variant II (Fig. 42, page 109 - the small, raised, beaded colony) did not give rise to a form identical with the original colony, but, after a similar number of subcultures it was derived from Variant I (a large, spreading form). Variant IAi (Fig. 41, page 109) was derived from Variant I after three subcultures and from Variant II after two subcultures.

Variant IAi (a large, spreading, finely wrinkled colony somewhat similar to the S form described by Petroff) and Variant II were injected into rabbits and guinea-pigs in order to determine any difference in virulence. As insufficient time has elapsed since the injection of the animals it has not been possible to include the results of the experiments in this report. If, however, differences of virulence prove to be associated with colony types details of the pathogenicity tests will be submitted in an addendum.

Correlation/

Correlation of Observations on Dissociation of the Human, Bovine and Attenuated Bovine (B.C.G.) Tubercle Bacilli.

The colonies of the human tubercle bacillus differed considerably from those of the bovine or attenuated bovine strains. They were dry and difficult to emulsify, and Variant IA (Fig. 39, page 109) was the only type so far observed which presented a moist, glistening appearance at all similar to that seen in colonies of the bovine strain. It has been suggested that B.C.G. in the course of attenuation has gradually developed certain characters of the human bacillus, and, on glycerine-egg it was observed to grow much more luxuriantly than on Dorset's egg medium. The colonies, however, did not resemble those of the human bacillus except in the case of the S form of B.C.G. (Fig. 16, page 78) which was somewhat similar in general appearance to Variant II2 (Fig. 49, page 112) of the human bacillus. The latter, however, was much more difficult to emulsify than the former. Therefore, as far as colony structure was concerned, it could not be said that B.C.G. showed any close relationship to the human bacillus.

Petroff, Branch and Steenken (1929) concluded from their studies of a number of strains of B.C.G. that the avirulence/

avirulence of the organism is due to the fact that prolonged subcultivation on glycerine-bile-potato medium eliminated the S or virulent forms as a result of dissociation to the R type. When a virulent strain of the organism was obtained they believed it was due to the presence in the culture of a few potentially virulent cells, possibly of the S type which on transfer to more suitable environmental conditions returned to the virulent state. Theoretically, therefore, it follows, that, if B.C.G. is an attenuated organism composed of R variants formed of large wax-like coils, the colonies in general should present a much coarser structure than those of a virulent strain of the bovine tubercle bacillus, and the S variants of B.C.G. should approximate to the colonies of the virulent organism. In other words, the round, smooth forms of the bovine bacillus would be extremely virulent and the R form of B.C.G. would be avirulent, and intermediate between these would be the somewhat similar, moderately virulent R variant of the bovine bacillus and S form of B.C.G. While one cannot justifiably compare the colonies of the attenuated and the virulent strains used in the above experiments since both were not derived originally from the same culture, it is of interest to note/

note in the observations recorded that, on the whole, B.C.G. formed colonies composed of larger and coarser coils than those of the virulent strain, the colonies of which were usually convex or spherical and smooth, or else large, rather flat and rough in structure. Several colonies of both strains, however, presented a certain similarity in their general structure. Variant ICI (Fig. 10, page 56) of the bovine tubercle bacillus closely resembled the R form (Variant II) of B.C.G. both being composed of large, wax-like coils which were emulsified only with difficulty. The flat, spreading S form of B.C.G. (Fig. 16, page 78) was similar in some respects to larger colonies of Variant II of the bovine bacillus (Fig. 42, page 109) and although some investigators report a high degree of virulence for this colony of B.C.G. the results recorded here fail to confirm such observations, only a low degree of virulence being exhibited by the S colonies or their subcultures. Variant II of the bovine bacillus although pathogenic to rabbits did not produce such extensive lesions as Variant I of the same strain. On the other hand, a smooth, glistening variant of B.C.G. which was similar to the smooth Variant I of the bovine bacillus and, in virtue of this fact should, theoretically/

theoretically at least, have possessed a high degree of virulence has been reported as avirulent (Kraus, 1929) and is considered an "intermediate" form by Petroff. The large and very rough types of colonies of B.C.G. shown in Figs. 33, 34 and 36 have not been observed among the colonies of the virulent organism. It is unlikely, however, that all possible types of variants have been recorded in either strain since recent subcultures were still resulting in forms not previously observed.

The three strains of slow-growing acid-fast organisms which have been examined all exhibited a remarkable variability in their colony structure, and, as far as could be judged from the limited number of subcultures which were made, none of the types showed any evidence of marked stability.

INVESTIGATION OF CERTAIN OTHER ACID-FAST BACILLI.

Because of the slow rate of growth of the human and bovine strains of the tubercle bacillus, a study of dissociation among these organisms must necessarily be extremely limited unless pursued for a considerable number of years. In order, therefore, to make a more thorough study of acid-fast organisms several rapidly growing strains were examined.

In 1930 Begbie reported moist and smooth, and dry and friable colonies in a strain of Clegg's "Bacillus leprae". After one subculture these remained true to form. Variants of the smegma bacillus were also observed. Kahn and Schwartzkopf (1932) working with a strain of "B. leprae" isolated in 1912 from a case of rat "leprosy" found that smooth forms on gentian-violet-egg medium dissociated to rough and rough to smooth without any special effort being made to induce the change. The experiments were carried out with single cell cultures.

"BACILLUS LEPRAE"

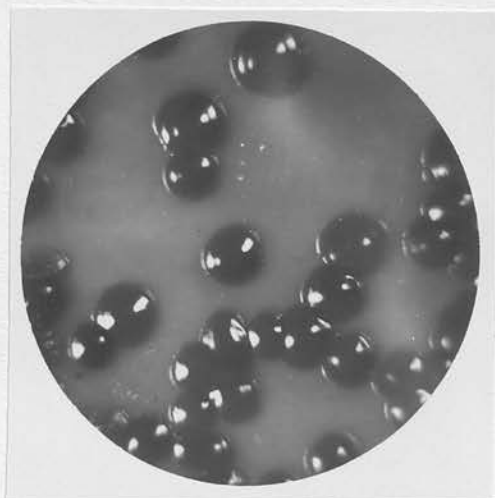


Fig. 50. Variant I.
14 days' growth. x 10.

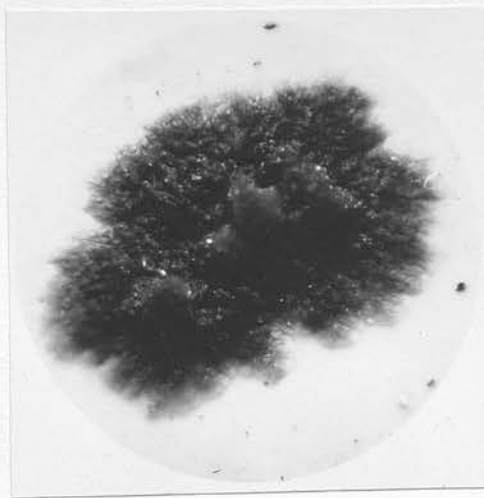


Fig. 51. Variant II.
20 days' growth. x 10.

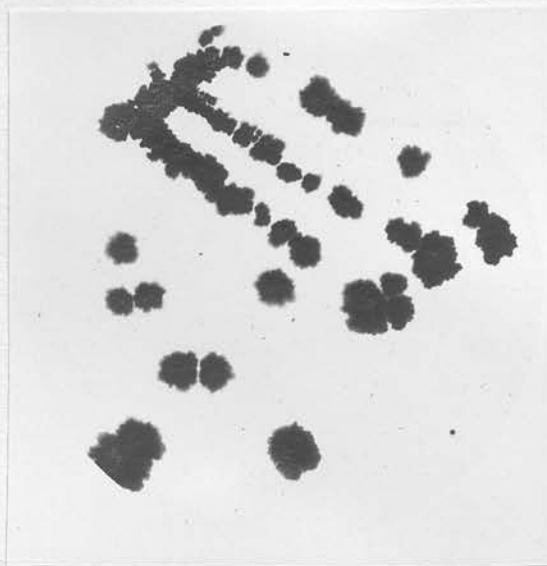


Fig. 52. Variant II. 20 days'
growth. Natural size.

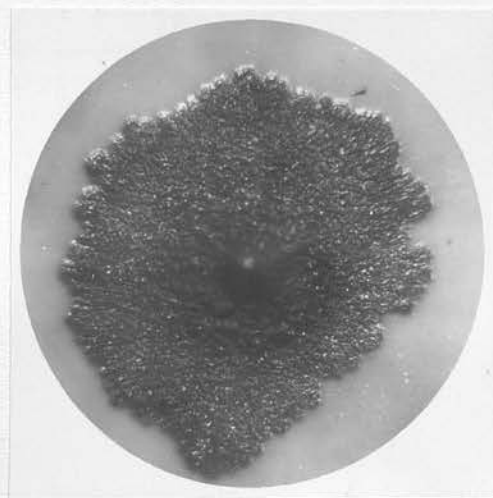


Fig. 53. Variant III.
20 days' growth. x 10.

"BACILLUS LEPRAE" (BRINCKERHOFF I.)Dissociation Experiments.

A strain of "Bacillus leprae" (Brinckerhoff I) was obtained from the National Collection of Type Cultures. It was characterized by a brilliant yellow pigment, and grew well on ordinary media, the growth being of a rather irregular glistening appearance.

When plated on gentian-violet-egg medium three types of colonies were observed:

Variant I. This variant was perfectly round, smooth convex, glistening and entire. It formed an even suspension in saline (Fig. 50).

Variant II. To the naked eye this colony appeared matt and the growth was emulsified only with difficulty. It was composed of numerous coils and furrows, was raised in the centre and sloped down to a rather irregular edge (Figs. 51 and 52).

Variant III. A large, flat, wrinkled, glistening periphery with a slightly raised centre characterized this form. It was, apparently, intermediate between the two variants just described (Fig. 53).

Growth in Bouillon.

In bouillon Variant I formed a uniformly turbid growth but after several weeks a pellicle developed. Variant II was characterized by a granular sediment with a clear fluid and heavy pellicle which on shaking fell/

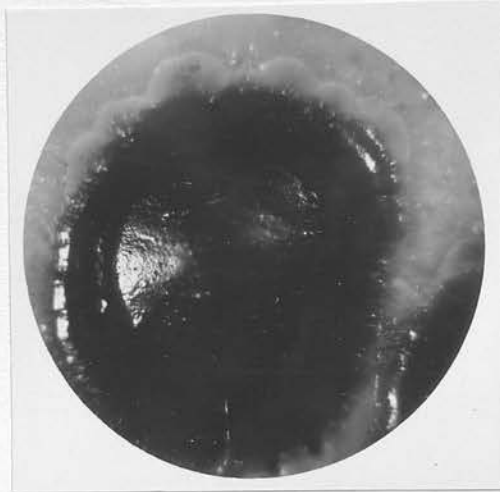
"BACILLUS LEPRAE"

Fig. 54. Variant I showing papillae and periphery of rough growth. 66 days' growth. x 10.

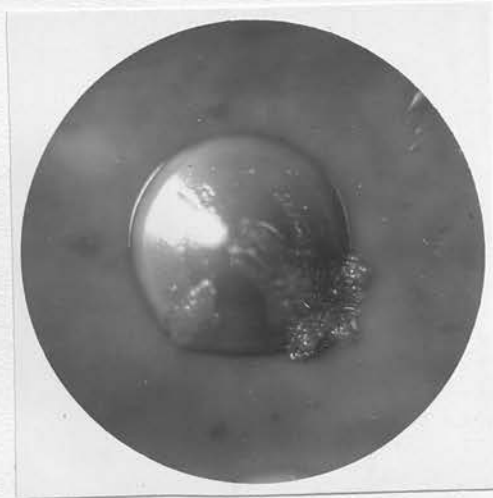


Fig. 55. Variant I showing segment of rough growth. 16 days' growth. x 10.

fell to the bottom of the container and was soon replaced by a second surface growth.

Stability of Variants on Gentian-Violet-Egg Medium.

Variants I and II when subcultured at intervals for a number of months appeared to remain relatively stable. From time to time, however, alterations in the growth were observed. They were most noticeable when Variant I was allowed to age. Small papillae and a peripheral growth similar to that of Variant II developed (Fig. 54) and this when replated gave typical, irregular, rough colonies. When growth from the centre of the colony was subcultured the resulting forms were identical with Variant I. Another form of variation was the appearance of a rough segment overgrowing the parent colony (Fig. 55). This was observed in a number of cases and appeared to arise from one or more papillae on the surface of the smooth colony. Subcultures from Variant III resulted in Variants I, II and III.

Growth of Variants on Uniform Media.

Up to this time subcultures had been made on different batches of gentian-violet-egg medium and it was thought that, possibly, slight variation in the constituents/

constituents was instrumental in causing changes in the types of growth. To determine, therefore, whether dissociation occurred when the variants were subcultured on slopes of a medium prepared from the same batch as that used for the first tube in the series, and also to see whether different media affected the stability of the type "typical" colonies of Variants I and II were emulsified in saline and inoculated on slopes of agar, heated-blood-agar, glycerine-egg, gentian-violet-egg, Loeffler's serum and bouillon. (Slopes were used in preference to plates as the latter could not be stored for a sufficient length of time.) The growth, which was examined with a plate culture microscope, was transferred every four days.

The results showed that both Variants underwent dissociation on all media. On agar Variant I dissociated most rapidly, the second subculture causing a partial change to an irregular, rough type of growth; and the change was complete by the eleventh transfer. On gentian-violet-egg medium variation was not observed until the ninth transfer. The first subculture of Variant II on Loeffler's serum and on heated-blood-agar resulted in some glistening, smooth growth, where-
:as/

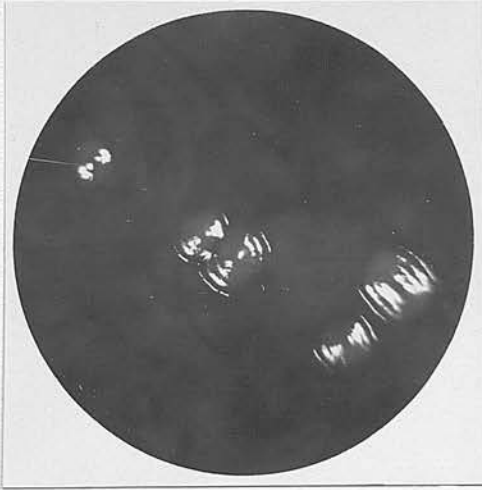
"BACILLUS LEPRÆ"

Fig. 56. Variant I showing concentric rings - Blood-agar. 8 days' growth. x 10.

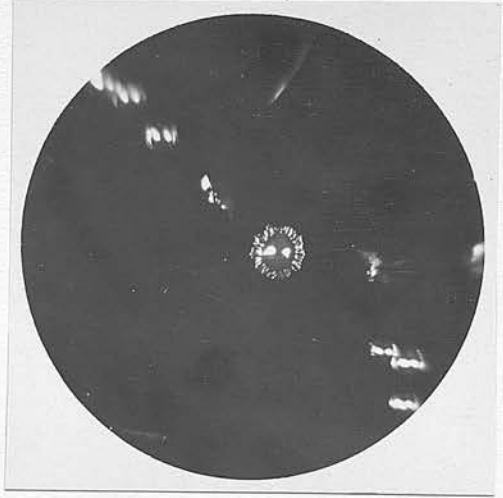


Fig. 57. Variant I which has developed a moist, irregular, glistening edge - Blood-agar. 8 days' growth. x 10.

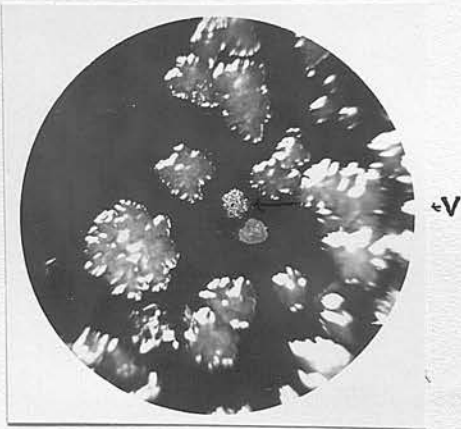


Fig. 58. Variants IV and V. Blood-agar - 8 days' growth. x 10.

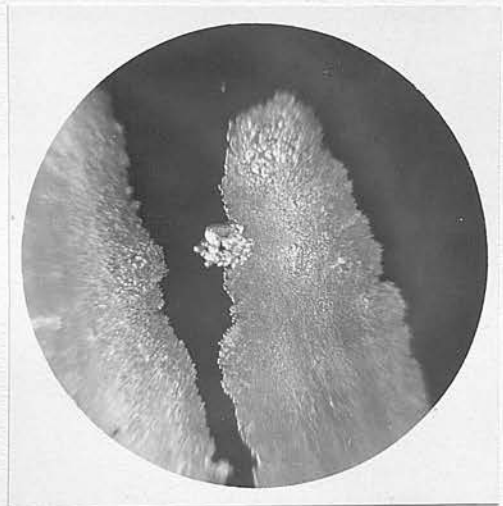


Fig. 59. Variant VI at edge of matt growth - Agar. 10 days' growth. x 10.

whereas on gentian-violet-egg medium a similar change was not observed until the thirteenth subculture.

Since only one batch of each medium was used it was hardly likely that variation could be attributed to differences in the environmental conditions, although possibly storage for a prolonged period might have caused the media to undergo some change.

Colony Variants Observed on Blood-Agar Medium.

It was noticed in the above experiment that on certain media Variants I and II exhibited a type of growth not previously observed and it was found that on blood-agar five distinct variants were clearly differentiated.

Variant I. Similar to that occurring on gentian-violet-egg. Both large and small forms developed and these when replated were found to be interchangeable. There were also smooth, glistening forms showing concentric rings (Fig. 56), or a moist, glistening, irregular edge (Fig. 57).

Variant IV. This was a fairly large, moist, glistening, easily emulsified colony, irregular in shape (Fig. 58).

Variant V. Fine, glistening coils characterized this variant. It was irregular in outline and difficult to emulsify (Fig. 58).

Variant VI. This colony was composed of larger coils than Variant V. They were glistening and did not form an even suspension in saline (Fig. 59).

Variant/

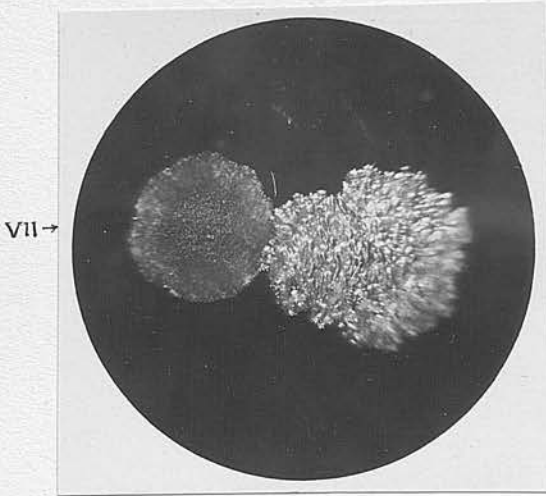
"BACILLUS LEPRAE"

Fig. 60. Variant VII.
Blood-agar. 8 days' growth.
x 10.

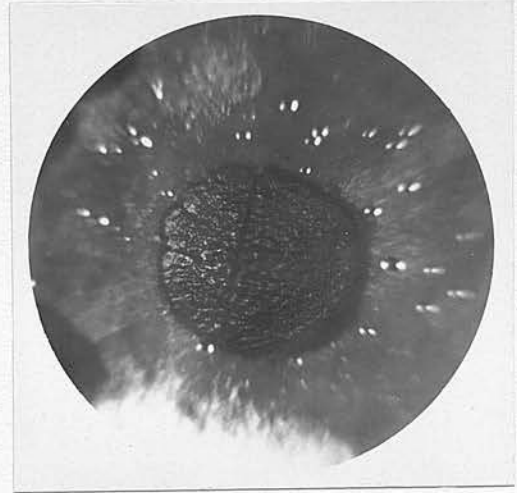


Fig. 61. Variant VIII.
Glycerine-egg medium.
31 days' growth. x 10.

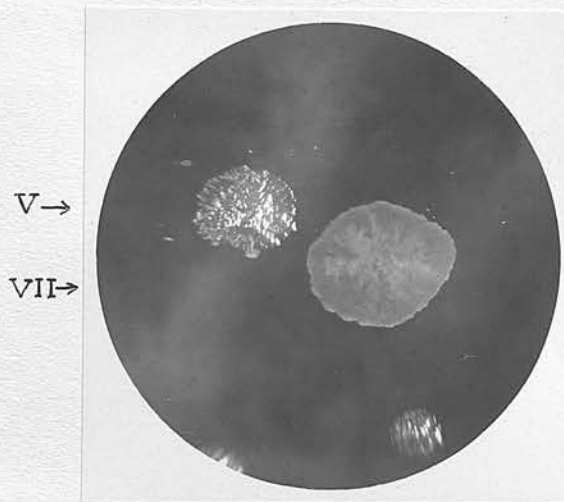


Fig. 62. Variants VII and V.
Heated-blood-agar. 10 days'
growth. x 10.

Variant VII. Unlike the previous forms this was matt, flat, usually round, composed of fine furrows and difficult to emulsify (Fig.60).

Colony Types Resulting from Subcultivation of Variants I and VII on Various Media.

A colony of each of the most extreme types observed on blood-agar - Variants I and VII - was picked off, emulsified in saline and inoculated on plates of agar, blood-agar, heated-blood-agar, Loeffler's serum, glycerine-egg and gentian-violet-egg medium. Different batches of media were used for every two or three subcultures. At intervals of four days, growth from one plate was streaked directly onto a second plate without being filtered and diluted. The colonies were examined and the results are summarized in the following table. One colony not previously described was observed on glycerine-egg medium.

Variant VIII. After several days' growth this colony was similar to Variant I but had a granular surface. As it aged it became flattened and the surface beaded (Fig.61).

It was also noticed that Variants V and VII on heated-blood-agar (Fig. 62) were of a finer structure than similar variants cultivated on blood-agar (Figs. 58 and 60).

Summary/

Summary of Dissociation Experiments with "Bacillus leprae" when cultivated on various media.

Colony Types resulting from Transfers of Growth at intervals of four days.

V A R I A N T I

Medium	1st	2nd	3rd	4th	5th	6th	7th
Agar	I	I, V	I (large and small)	I (large and small)	I (large and small)	I	I
Blood-agar	I	I (large and small)	I (large and small)	I (large and small)	I (large and small)	I (large and small)	I
Heated-blood-agar	I	I (large and small)	I (becoming slightly irregular)	I (large and small)	I (large and small)	I (small) I (large with matt surface) VI	I (small, slightly irregular)
Loeffler's serum	I (with thin, matt veil) I (convex colony with granular surface)	I (with thin, matt veil) I (with edge of glistening coils)	I (convex colony with rough surface)	I (with uneven surface)	VII (some irregular glistening forms)	VII (convex colony with granular surface)	-
Gentian-violet-egg	I	I	I	I	I (with slightly matt surface)	I (and areas of II)	I (slightly granular or matt) II
Glycerine							

VARIANT I continued:

<u>Medium</u>	<u>1st</u>	<u>2nd</u>	<u>3rd</u>	<u>4th</u>	<u>5th</u>	<u>6th</u>	<u>7th</u>
Glycerine-egg	VIII IV	VIII I (with irregular, glistening edge)	VIII	VIII	VIII	VIII	I I (with irregular centre)
<u>Medium</u>			V A R I A N T	VII			
Agar	<u>1st</u> VII, VI	<u>2nd</u> VII, VI	<u>3rd</u> VII, VI	<u>4th</u> VII, V	<u>5th</u> VII, VI, V	<u>6th</u> VII, V	<u>7th</u> VII, VI
Blood-agar	VII, VI	VII, V	VII, V	VII, V	VII, V	VII, VI, V	VII, V
Heated-blood-agar.	VII, V	VII, VI, V	VII, V	VII, V	VII, V	VII, V	VII, V
Loeffler's serum	I, I (slightly irregular) I (with thin veil of matt growth)	VII I (with granular surface)	VII (convex with matt surface)	VII, I	VII, I (with thin veil of matt growth)	I (with thin veil of matt growth)	VII (with thin veil of matt growth)
Gentian/							

VARIANT VII continued:

<u>Medium</u>	<u>1st</u>	<u>2nd</u>	<u>3rd</u>	<u>4th</u>	<u>5th</u>	<u>6th</u>	<u>7th</u>
Gentian-violet egg.	II	II	II	II	II	II	II
Glycerine-egg.	II	II	II	II VIII	VIII VIII (with more irregular edge)	-	-

It is evident from the preceding table that when examined after four days on blood-agar Variant I remained relatively stable for seven transfers. On other media varying degrees of dissociation were observed. Presumably colonies similar in appearance do not possess the same degree of stability since, in the previous experiment when subcultured on agar slopes Variant I yielded a mixture of rough and smooth growth by the second transfer, and by the eleventh the smooth growth had been entirely eliminated. While subcultures of Variant VII showed colonies similar to the parent type these were always associated with other variants and on gentian-violet-egg medium Variant II was the only form observed. Previously it had been observed that after thirteen transfers on gentian-violet-egg slopes this variant reverted to a smooth, glistening form.

On Loeffler's serum it was found that either Variant II from gentian-violet-egg medium or Variant VII dissociated rapidly to large, round, moist, glistening, smooth forms very similar to Variant I or to forms which, although convex, had a granular surface. Frequently the glistening colonies were covered with a/
a/

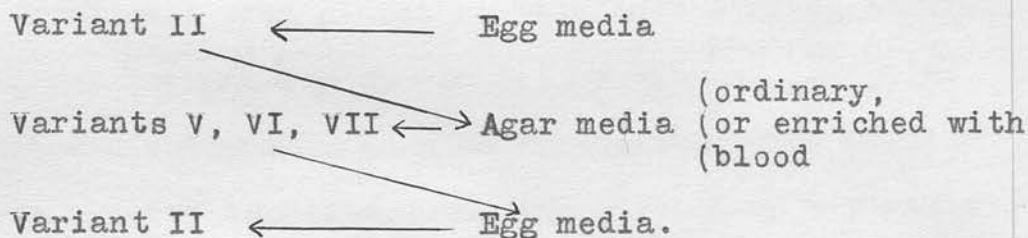
a thin veil of matt growth and as the colony enlarged this gradually broke into pieces which remained as small matt areas on the glistening surface. These colonies were easily emulsified.

The Effect of Transferring Variants from One Medium to Another.

During the course of the above experiment different colony types were frequently removed from one medium and inoculated on to the various other media, and it was found that Variant I when transferred from gentian-violet-egg medium frequently underwent variation. Sometimes it was only a slight change in the appearance of the surface or form of the colony, at others a complete transformation to a coarser type. When a colony was round and convex in shape but characterized by a matt or granular surface, a type of growth similar to that of Variants II or III usually extended from it as it aged.

Since Variant VII on gentian-violet-egg medium resulted in colonies of Variant II but on agar, blood-agar and heated-blood-agar showed matt and glistening forms (both of which were difficult to emulsify) and since these two forms had not been observed on gentian-violet/

gentian-violet-egg, it seemed probable that the latter medium was unsuitable for differentiating these types. Glistening and matt forms were removed from agar, blood-agar and heated-blood-agar and replated on the various media. They were interchangeable except on the two egg media where the colonies were similar to Variant II. Conversely when Variant II from glycerine-egg or gentian-violet-egg medium was replated types similar to the parent form developed on the egg media but on the agar and enriched agar plates both glistening and matt forms resulted. This may be represented diagrammatically as follows:



There appeared to be a close relationship between the glistening Variants V and VI and the matt Variant VII since, as the glistening forms aged, a broad, matt periphery developed and, as the matt forms aged, areas of glistening growth became visible in the colony and gave the appearance of a thin veil of matt growth over a glistening surface.

The/

The Formation of Papillae.

Subcultures of Variant I on glycerine-egg medium were outstanding in their ability to form papillae. The supergrowth appeared within a few days and in some cases formed a protruding growth which as it lengthened bent to one side (Fig. 63) or curved round and rejoined the parent growth. When it was plated on gentian-violet-egg, glycerine-egg and blood-agar colonies of Variant I resulted. On heated-blood-agar the colonies were glistening and irregular and on agar small, irregular and matt. Occasionally on glycerine-egg medium large, irregular coiled papillae were observed, and when plated on gentian-violet-egg medium, besides Variant I a smooth colony with an irregular centre developed. When an irregular, rough papilla from gentian-violet-egg was subcultured on a plate containing the same medium colonies of Variant II resulted. Papilla formation on Variants II, V, VI and VII was not observed.

Variation in Pigment Formation.

Pigment formation was delayed if tightly sealed plates or tubes were not exposed to the air a few days after inoculation. Occasionally light coloured areas of/

"BACILLUS LEPRÆ"

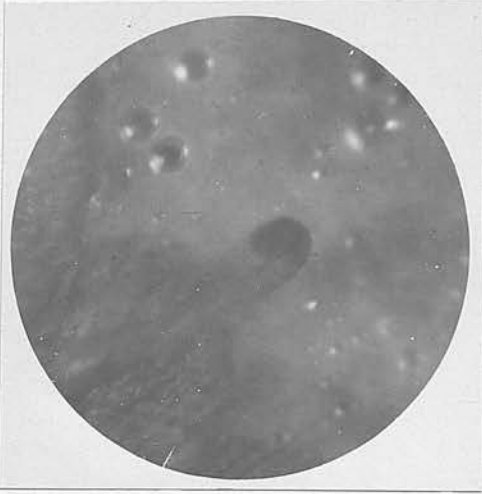


Fig. 63. Long supergrowth.
Glycerine-egg medium.
12 days' growth. x 10.



Fig. 64. Gentian-violet-egg
medium. 10 days' growth.
x 1,000.

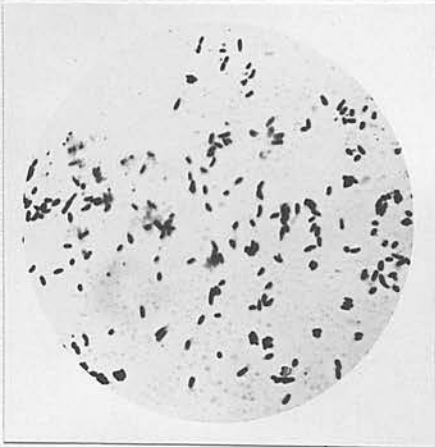


Fig. 65. Agar medium.
10 days' growth. x 1,000.



Fig. 66. Loeffler's serum.
7 days' growth. x 1,000.

of growth were observed and in one instance a segment of a much darker shade than the rest of the colony developed. When subcultured some of the resulting colonies were of the usual colour while others were darker.

Variation in Morphology.

On gentian-violet-egg medium the bacilli of Variants I and II were considerably longer (Fig. 64) than those observed on agar, heated-blood-agar or glycerine-egg (Fig. 65). Subcultures of these two forms on Loeffler's serum and bouillon also resulted in long, slender organisms which on the former medium exhibited marked pleomorphism (Fig. 66). Usually the "rough" variants, II, V, VI and VII exhibited shorter organisms than the "smooth" Variant I.

Growth of Variants I, IV, V and VII in Fluid Medium.

Since marked stability of colony types was not observed on solid media Variants I, IV, V and VII from blood-agar plates were inoculated into 150 c.c. amounts of 2 per cent. glycerine bouillon. After one month subcultures were made on blood-agar and gentian-violet-egg plates, with the following results:

Growth/

Variants.	Growth in 2 per cent. glycerine bouillon.	Colonies on blood-agar plates.	Colonies on gentian-violet-egg plates.
I	Turbid at first. Later a pellicle and slight granular sediment developed and the fluid became clearer.	I with thick, glistening, periphery. I with thin, matt periphery. V, VII. VI with matt periphery.	II
IV	Turbid - a few, thin areas of surface growth.	IV IV (edge rather dull).	I, IV. IV with granular, glistening centre. IV with matt edge.
V	Heavy pellicle, clear fluid, granular sediment.	V VII	II
VII	No surface growth; clear fluid. Some granular growth at foot of bottle.	V VII	II

From this experiment it may be seen that Variant I dissociated to a variety of types, that Variant IV was relatively stable and that Variant V and VII again proved to be interchangeable. The occurrence of Variant IV on gentian-violet-egg plates has been observed only rarely.

Serum/

Serum Reactions.

When the study of this organism was first begun and it was thought that Variant I and Variant II were relatively stable on gentian-violet-egg medium rabbits were immunized against the respective types. The animals were inoculated once a week with graded doses of growth from seven day cultures. In order to prepare a fine suspension of Variant II it was necessary to emulsify a large amount of growth in saline, throw down the clumps by centrifuging for about half a minute and then pipette off the supernatant fluid and dilute it to the required turbidity. Variant I formed an even suspension and did not require this treatment.

Agglutination and Agglutinin-Absorption Tests showed the following results:-

Serum/

"B. leprae"
Agglutination and Agglutinin-Absorption Tests
 with Variants I and II.

Serum dilutions	1: 60	1: 120	1: 240	1: 480	1: 960	1: 1920	Control
<u>Variant I antiserum</u>							
vs.							
Variant I	+++	+++	++++	+++	+++	+++	-
Variant II	+++	++++	+++	+	-	-	-
<u>Variant I antiserum absorbed by</u>							
<u>Variant I</u>							
vs.							
Variant I	++	++	++	-	-	-	-
Variant II	-	-	-	-	-	-	-
<u>Variant I antiserum absorbed by</u>							
<u>Variant II</u>							
vs.							
Variant I	+++	+++	+++	++	+	-	-
Variant II	-	-	-	-	-	-	-
<u>Variant II antiserum</u>							
vs.							
Variant I	++++	++++	++++	++++	++	-	-
Variant II	++++	++++	+++	+++	++	-	-
<u>Variant II antiserum absorbed by</u>							
<u>Variant I</u>							
vs.							
Variant I	-	-	-	-	-	-	-
Variant II	+	+	-	-	-	-	-
<u>Variant II antiserum absorbed by</u>							
<u>Variant II</u>							
vs.							
Variant I	+++	+++	-	-	-	-	-
Variant II	-	-	-	-	-	-	-

It/

It was found that Variant I antiserum agglutinated Variant I and Variant II, but to a less degree, whereas Variant II antiserum agglutinated both Variants II and I to the same degree. Variant I absorbed the agglutinins for Variant II and for Variant I, though incompletely, from Variant I antiserum and the agglutinins for Variant I and also, though incompletely, for Variant II from Variant II antiserum.

Variant II absorbed the agglutinins for Variant II from Variant I antiserum and the agglutinins for Variant II and also, though incompletely, for Variant I from Variant II antiserum. The absorption of agglutinins for Variant I from Variant I antiserum was slight.

These tests showed the difference in the antigenic character of the two variants.

Several/

Several attempts have been made to induce dissociation more rapidly by growing the variants in their homologous immune sera, and also to stabilize them by growing in immune serum prepared against another colony type. So far no satisfactory results have been obtained since the organisms, although growing in ordinary bouillon, tend to die out after two or three subcultures in bouillon containing ten per cent. of the respective antisera.

Summary of Observations.

From this study of Brinckerhoff's "leprosy bacillus" it may be concluded that variation occurs in colony types, growth in bouillon, antigenic structure and morphology, and that some media are more suitable than others for differentiating the colony types. The most frequently occurring forms on the various media when inoculated with Variants I and VII were:

Variant/

	<u>Variant I</u>	<u>Variant VII</u>
Agar	I	V, VI, VII
Blood-agar	I	V, VI, VII
Heated-blood-agar	I	VII, V
Loeffler's serum	I, VII	I, VII
Gentian-violet-egg	I, II	II
Glycerine-egg	VIII, I	II

While complete stability of any one of the types tested was not observed, it was noticed that variation occurred more slowly on some media than on others. Variant I remained relatively stable on enriched agar media, and, in one experiment on agar, but in a previous case it dissociated rapidly when cultivated on this medium. Variant II when plated on agar media resulted in three different variants and these when subcultured on egg media again resulted in colonies of Variant II. Loeffler's serum induced dissociation of the rough Variants II and VII to a smooth form more rapidly than any of the other media examined, and the organisms exhibited marked pleomorphism. It was not found possible to obtain cultures of Variant VII without the closely associated Variants V or VI.

THE FISH TUBERCLE BACILLUS.Dissociation Experiments.

A strain of fish tubercle bacillus ("Cobbett"), obtained from the National Collection of Type Cultures was used in the following experiments. On ordinary media it formed a white, moist, glistening and easily emulsifiable growth. When subcultured on gentian-violet-egg medium it grew luxuriantly and absorbed the dye from the medium, the growth thus becoming violet coloured. Several attempts were made to induce dissociation by plating but the results were unsatisfactory. When, however, the organism was grown on Long's synthetic medium (Tubercle, 1924, VI, 128) and was then subcultured on gentian-violet-egg plates two distinct types developed, the one violet, the other white. These will be referred to as Variants I and IX respectively.

Subsequent plating revealed further variation within the types. The most frequently occurring variants observed are summarized as follows:

Variants/

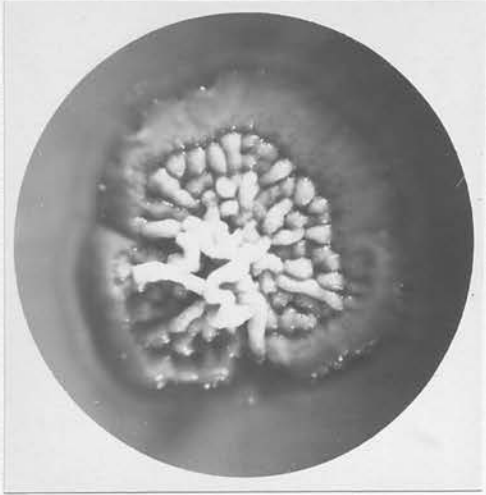
THE FISH TUBERCLE BACILLUS

Fig. 67. Variant I (violet).
14 days' growth. x 5.

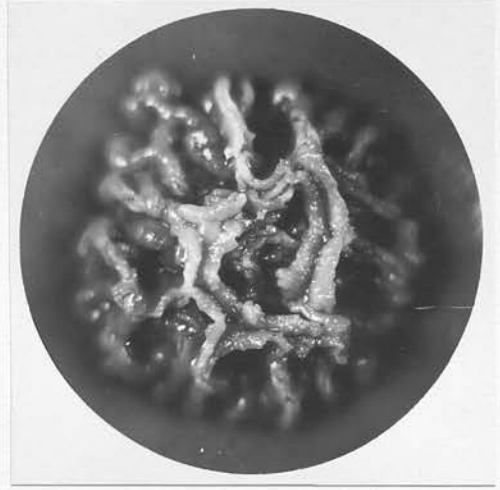


Fig. 68. Variant II (violet).
14 days' growth. x 10.

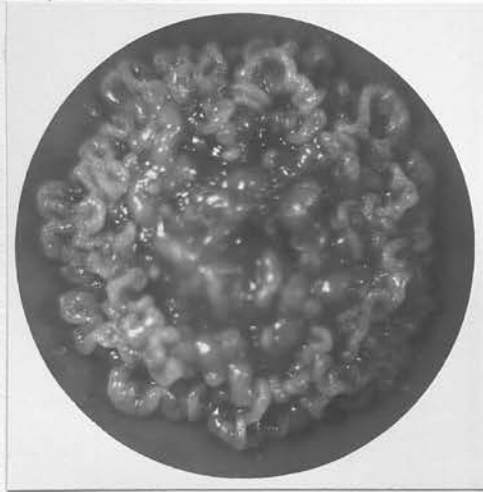


Fig. 69. Variant III (violet).
Variant VIII (white). 14 days'
growth. x 10.

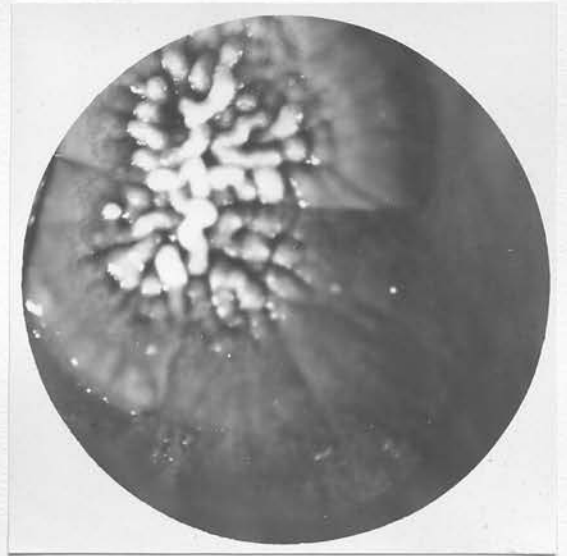


Fig. 70. Variant I (violet)
24 days' growth. x 5.

Variants Taking up Dye from Gentian-Violet-Egg Medium.

Variant I. This colony was round or irregular and had a central coiled area. The coils were either flat or raised above the rest of the colony and they extended from the centre outwards to a thick, opaque, glistening, smooth border. From this a narrow fimbriate periphery developed (Fig. 67).

Variant II. In this variant the coils were high and formed a crown-like structure (Fig. 68). As the colony aged it increased in size and became somewhat similar to Variant I, but the coils were coarser and there was no narrow fimbriate periphery.

Variant III. This variant was round and convex and was characterized by a mass of coils which merged into an uneven glistening centre (Fig. 69).

A further variation was observed when Variant I developed a white, matt segment (Fig. 70). It was difficult to emulsify and further subcultivation resulted in white variants of the following structure:

Variants not Taking up Dye from Gentian-Violet-Egg Medium.

Variant IV. This variant was similar in structure to Variant I but the surface was usually matt and slightly irregular and there was no fimbriate periphery. The edge was the same as that shown in Fig. 71 but the central coils were thicker and less numerous and resembled those in Fig. 70. In some instances this form developed a very wide border (Fig. 72).

Variant V. In general Variant V was the same as Variant II but did not take up the dye from the medium (Fig. 73). The edge varied in width and sometimes a spreading border developed/

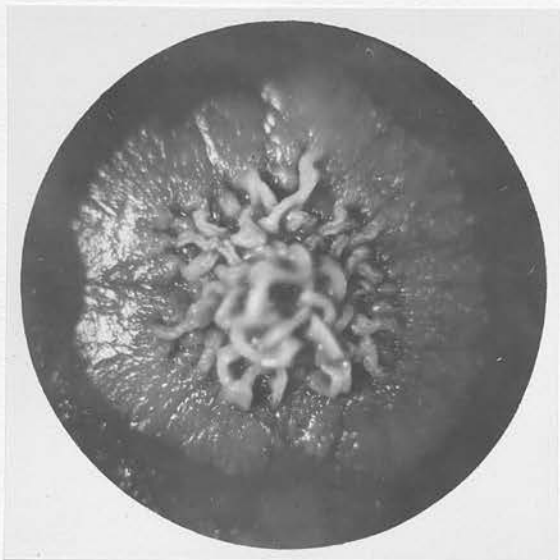
THE FISH TUBERCLE BACILLUS

Fig. 71. Colony somewhat similar to Variant IV (white). 14 days' growth. x 10.

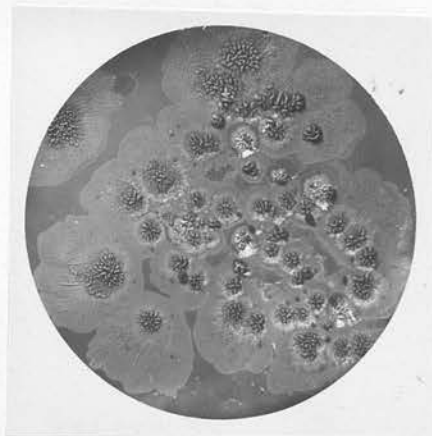


Fig. 72. Variant IV (white) with unusually broad periphery. 35 days' growth. Natural size.



Fig. 73. Variant V (white) 18 days' growth. x 10.

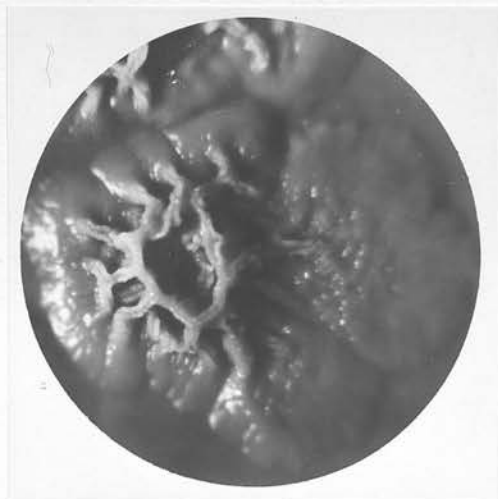


Fig. 74. Variant V (white) showing spreading border. 18 days' growth. x 7.

developed (Fig. 74) or the coils were replaced by a single ridge of growth surrounding the central cavity.

Variant VI. This colony was flat and characterized by three or four ridges extending from the centre to a peripheral ridge. The edge was glistening and irregular (Fig. 75).

Variant VII. This variant was raised and had a central cavity surrounded by smooth, glistening growth which sloped down to a narrow, slightly irregular edge (Fig. 76).

Variant VIII was identical with Variant III but did not take up the dye from the medium (Fig. 69).

Variant IX which was observed after growing the undissociated culture on Long's synthetic medium and then plating on gentian-violet-egg medium was also white and difficult to emulsify.

Variant IX. This was a high, round, convoluted colony, the coils extending in every direction over the surface and to the very edge of the colony. It grew rapidly until it reached a size of two or three centimetres (Fig. 77). When it aged it became orange in the centre and the coils sloped to the edge which became flat and glistening and took up the dye from the medium (Fig. 78).

Stability of Colony Types.

None of the types examined exhibited absolute stability. In general, however, violet forms were derived from colonies of this colour and white variants from white forms. Occasionally a violet colony formed a white segment, as already described, and conversely

a/

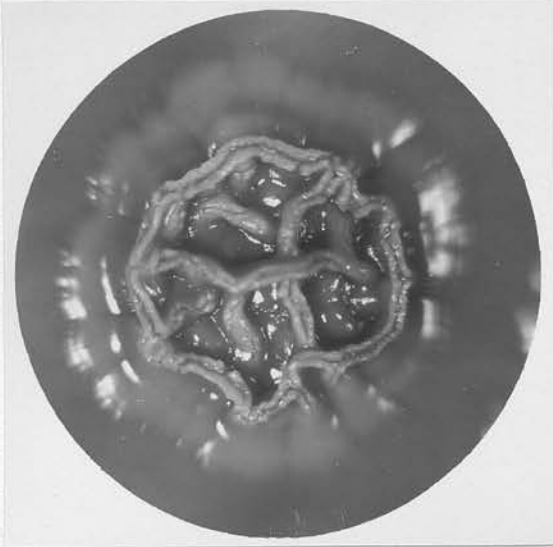
THE FISH TUBERCLE BACILLUS

Fig. 75. Variant VI (white).
34 days' growth. x 10.

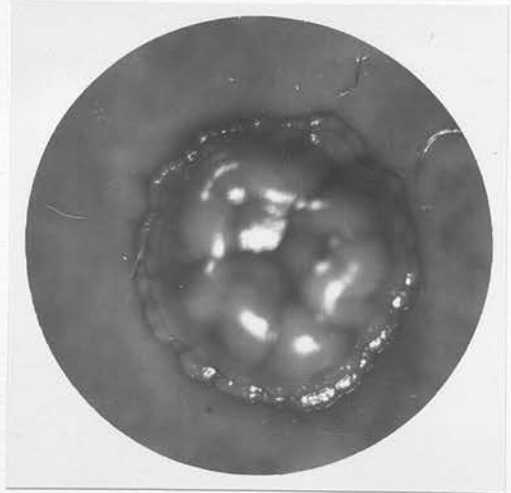


Fig. 76. Variant VII (white).
34 days' growth. x 10.

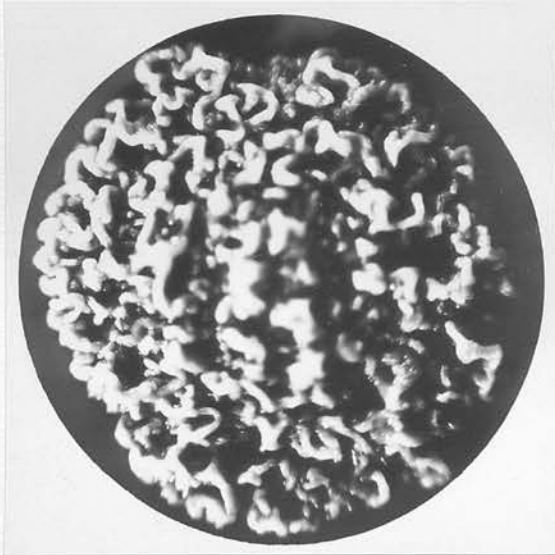


Fig. 77. Variant IX.
24 days' growth. x 5.



Fig. 78. Variant IX showing
border similar to Variant I.
61 days' growth. x 10..

a white colony formed a violet segment. More frequently when the white colony acquired the ability to take up the dye from the medium traces of colour were observed in what had previously been white growth; the change therefore was not always abrupt. When such an area was replated both violet and white colonies resulted. As a rule the difference in colour was quite distinct, but on two or three occasions variants of an indefinite, pale violet shade were observed.

The most commonly occurring white variant was that shown in Fig. 73. (page 147) but after five subcultures a more coiled form resulted (Fig. 79).

When a broad white border (Fig. 72, page 147) was plated, besides forms somewhat similar to IV and V there also developed Variant VII (Fig. 76). The colonies arising from subcultivation of this type were mainly characterized by a central, convex, violet dome surrounded by a dull, white periphery, or else were rather similar to Variant IV.

From time to time it was observed that when a batch of plates received the same inoculum one plate developed one type of colony and another a different type, while on a third both types occurred. The plates were apparently identical in depth of medium, and/

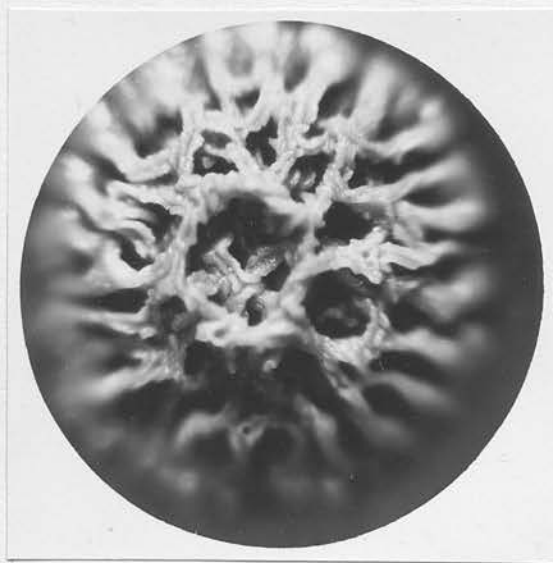
THE FISH TUBERCLE BACILLUS

Fig. 79. Variant V after five subcultures - 18 days' growth. x 10.

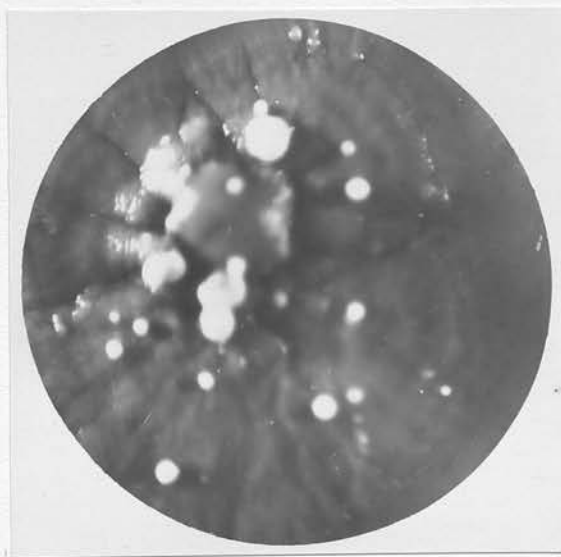


Fig. 80. Variant V showing papillae formation. 65 days' growth. x 10.

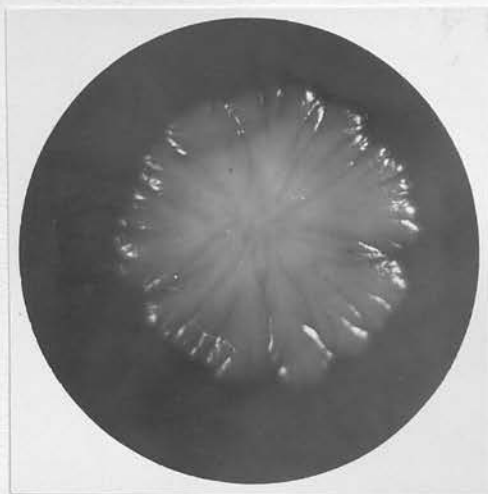


Fig. 81. Glistening variant on agar - 14 days' growth. x 10.

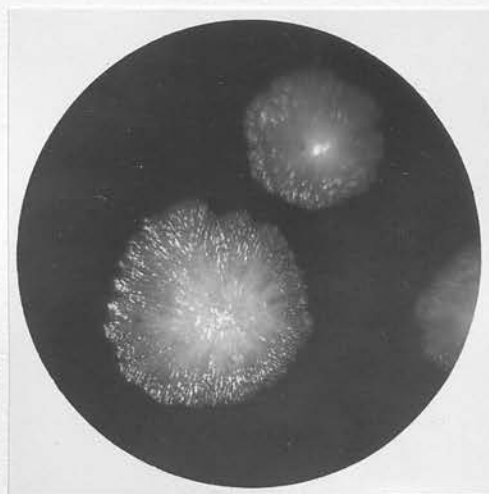


Fig. 82. Finely furrowed variant on agar - 14 days' growth. x 10.

and concentration of dye and moisture, and therefore environmental conditions were not wholly responsible, and it was concluded that some other factor (or factors) was involved. This peculiarity was observed in various other organisms but so far no satisfactory explanation for its occurrence has been forthcoming.

The Formation of Papillae.

Papilla formation on both violet and white colonies was frequent (Fig. 80). They remained as glistening or matt spheres or became extremely coiled, spreading over the parent growth and becoming orange as they aged. When one of these papillae was replated colonies similar to Variants III and VIII developed on one plate, the only difference being that Variant III took up the dye from the medium and Variant VIII did not, and on another plate the variant shown in Fig. 71 was observed. Agar plates inoculated with the same filtrate as the gentian-violet-egg plates showed two different types of colonies (Figs. 81 and 82) and by subsequent plating it was found that the glistening, opaque, white form corresponded to the violet colony on gentian-violet egg. When Variants III and VIII (Fig. 69, page 145) were subcultured the plates developed/

developed coiled colonies somewhat flatter than the original and on each batch of plates a few forms were of a colour other than the parent type. Variant VI (Fig. 75, page 149) was derived from Variant VIII.

Variant VIII (Fig. 69, page 145 - 14 days' growth) and Variant IX (Fig. 78, page 149 - 61 days' growth) presented a similar structure. When the violet border shown in Fig. 78 was subcultured colonies of Variant I resulted. Papilla formation was never observed on Variants III, VIII and IX.

Variant IV (Fig. 71, page 147) and Variant IX (Fig. 77, page 149) were both obtained by plating a papilla from a white colony, and so far this has been the only occasion that Variant IX has been obtained since it was observed after growth in Long's medium. Very possibly, therefore, its presence was due originally to secondary growth formation in the liquid medium.

Variation in Morphology.

The violet colonies were emulsified readily in physiological salt solution. When examined microscopically the arrangement of the cells was irregular, in pairs or sometimes in short chains. The white colonies were difficult to emulsify and the organisms formed irregular clumps or groups of cells in parallel formation/

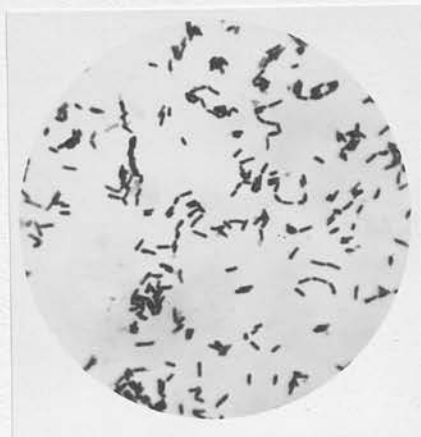
THE FISH TUBERCLE BACILLUS

Fig. 83. Short organisms.
14 days' growth. x 1,000.



Fig. 84. Short and long
organisms. 14 days' growth.
x 1,000.



Fig. 85. Colony composed of
long cells. 18 days' growth.
x 10.



Fig. 86. Long organisms.
5 days' growth. x 1,000.

formation. The cells themselves varied greatly in size but so far it has not been found possible to correlate short or long organisms with particular colony types. In general the bacilli of violet colonies were short (Fig. 83) but occasionally long cells were also seen among the smaller forms. This was frequent in the case of white colonies where extremely minute cells and clumps of long organisms occurred side by side (Fig. 84). Microscopic preparations made from Variants III and VIII (Fig. 69, page 145), from the colony shown in Fig. 85, and from some papillae were composed almost entirely of long organisms (Fig. 86). As far as could be ascertained the only characteristics common to the growths from which these long cells were derived, were a somewhat indefinite structure and a rather moist consistency.

Growth in Fluid Medium.

Although violet colonies were emulsified much more readily than white variants both agglutinated in 0.1 per cent NaCl. The following variants were grown for twelve weeks in 2 per cent. glycerine bouillon and then subcultured on gentian-violet-egg medium:

Variant/

<u>Variant</u>	<u>Growth in 2 per cent. glycerine bouillon</u>	<u>Growth on gentian-violet-egg medium.</u>
I	Pellicle, clear fluid, flaky sediment.	II, V (very few of Variant V).
II	Pellicle, clear fluid, flaky sediment.	II, V - colonies frequently spread and become rather flat.
V	Pellicle, clear fluid, granular sediment.	V, II (very few of Variant II).
III	Pellicle, turbid, flaky sediment.	II, V - colony centres very coiled.
VIII	Pellicle, turbid, flaky sediment.	II, V - colony centres very coiled.

When shaken the pellicle fell to the bottom of the container and a thick ring was left round the surface of the medium. Although all the variants derived from plating the growth from glycerine bouillon cultures were classified broadly as II or V, they showed a certain amount of variation from the "typical" forms. Both violet and white colonies were observed in every case. The variants derived from III and VIII were similar in their colony structure.

Pathogenicity/

Pathogenicity Experiments.

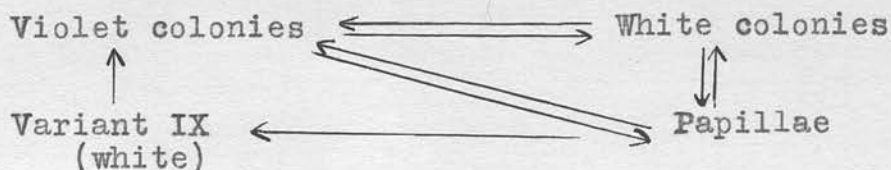
Variants I, V and VIII were injected intramuscularly into rainbow and brown trout, the former receiving a dose of 10 mgm., and the latter 5 mgm.. The two fish injected with Variant VIII and the rainbow trout injected with Variants V and I all developed local lesions and these were still persisting when the former were killed after eleven weeks and the latter after fifteen weeks. The lesions of the rainbow trout injected with Variants I and VIII were characterized by slight necrosis and some fibrous tissue, and the lesions in the other two by fibrous tissue only. (The brown trout injected with Variant I did not present any obvious lesion and the one injected with Variant V died as a result of the injection.) The fish when killed were all in good condition, organisms were isolated from the lesions and the colonies developing on gentian-violet egg plates were somewhat similar to the variants which had been injected, the only differences being that Variant I had partly dissociated to Variant II and one colony showed a white segment. Variant VIII had a more granular centre than previously. Both white and violet colonies were isolated from the fish injected/

injected with Variant V.

As far as could be judged from such a limited experiment none of the variants was highly virulent and there was no marked dissociation of types to a "smooth" form after growth in vivo.

Summary of Observations.

The fish tubercle bacillus, like the other acid-fast organisms already described, undergoes variation in colony structure, growth in bouillon and morphology. From the work that has been done so far the results might be represented diagrammatically on the ability or inability of the variants to take up dye from gentian-violet egg medium:



The various types were interchangeable but Variant IX, which was obtained by plating a papilla, was never observed to form supergrowths. As it aged, however, a peripheral growth of Variant I developed. In general the violet colonies were more easily emulsified than the white forms.

FRIEDMANN'S BACILLUS

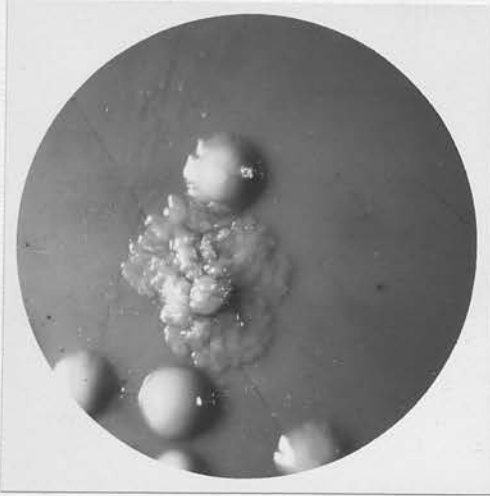


Fig. 87. 12 days' growth.
x 10.

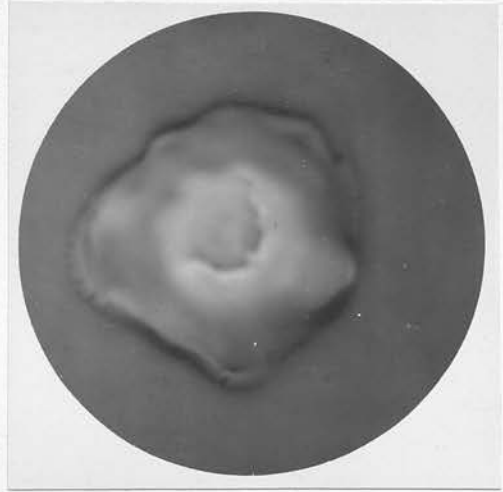


Fig. 88. 16 days' growth.
x 10.

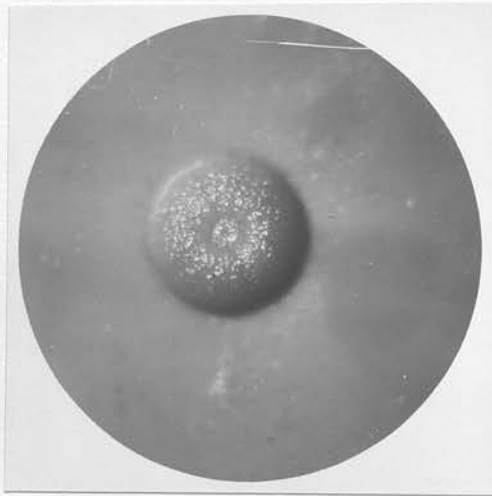


Fig. 89. 21 days' growth.
x 10.

THE TORTOISE TUBERCLE (FRIEDMANN'S BACILLUS)

and

MYCOBACTERIUM RUBRUM.

Besides the organisms which have already been considered, a less detailed study has been made of Friedmann's bacillus and Mycobacterium rubrum. Both these organisms exhibited a wide variety of colony types, but in order to devote more attention to the strains already described, the investigation of these two species was discontinued. Several points of interest, however, may be mentioned.

During the short time they were under observation both strains exhibited types ranging from perfectly round, smooth, convex, entire, glistening and easily emulsified forms to others which varied in their degree of "roughness" from coiled glistening variants to (in the case of Friedmann's bacillus) flat, wrinkled and matt colonies. Some of the variations occurring in the tortoise tubercle bacillus are represented in Figs. 87 to 91. The colony shown in Fig. 88 was of particular interest. It occurred periodically, was of a moist, glistening appearance and butyrous consistency, and/

FRIEDMANN'S BACILLUS

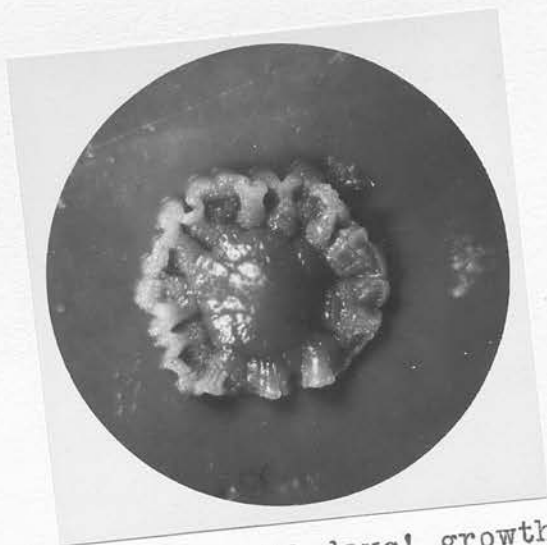


Fig. 90. 39 days' growth.
x 10.

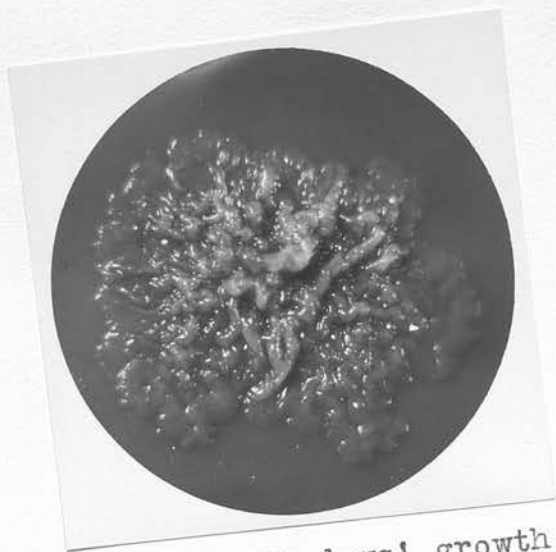


Fig. 91. 12 days' growth.
x 10.

and usually increased in size until it was larger than any of the other variants on the plate. It was unstable.

Two colonies of Mycobacterium rubrum are shown in Figs. 92 and 93. In this organism continued subcultivation seemed to increase the complexity of the colonies which at first were relatively smooth, but eventually exhibited types such as that shown in Fig. 93. An interesting point in connection with the organism was its ability to throw off variants characterized by a dark red pigment instead of the commonly observed salmon pink colour. This variation occurred abruptly in the form of a segment and when replated usually remained true to type; occasionally, however, a pale variant was observed. The colonies of the dark type were either smooth or coiled and exhibited differences in structure corresponding to those seen in the less pigmented strain.

While there seemed to be a gradual trend towards the "rough" type of growth in Mycobacterium rubrum no such sequence was observed in the tortoise tubercle bacillus and although a considerable number of types were subcultured they were not found to be stable; but, as has already been mentioned, the study of these organisms/

MYCOBACTERIUM RUBRUM

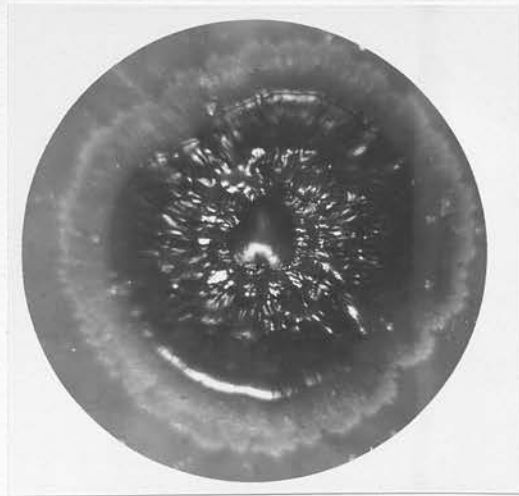


Fig. 92. 75 days' growth.
x 10.



Fig. 93. 58 days' growth.
x 10.

organisms was rather limited. It was, however, sufficient to emphasize once again that colonies of acid-fast organisms may exist in a wide variety of different forms.

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Since it has been found that the results obtained in the study of diphtheroid organisms are similar to those obtained among acid-fast organisms, a discussion of the foregoing section will be left over until the observations dealing with the diphtheroids have been recorded.

THE DIPHThEROID BACILLI.

Owing to the close association between the diphtheria bacillus and other diphtheroid bacilli, the subject of variation among these organisms has long been of interest to bacteriologists. Roux and Yersin (1890) believed that avirulent diphtheroids which occur in the throats of healthy individuals and are morphologically similar to the Kleb's-Loeffler bacillus are attenuated diphtheria bacilli and under certain conditions regain their virulence. A wide variety of different colony types was observed by Bernhardt and Paneth (1913) who concluded from their observations that no clear distinction could be made between the true diphtheria bacillus and other diphtheroids. The variants changed from one type to another in vivo as well as in vitro. Later Bernhardt (1915) pointed out that a type which occurred regularly on one medium did not necessarily occur regularly under other conditions and that on Loeffler's serum cultural differences disappeared. Baerthlein (1913) did not describe as many variants as Bernhardt but found them more stable, prolonged subcultivation in bouillon being necessary before/

before reversion to the original types occurred.

Two strains of diphtheria were studied in detail by Cowan (1927) who observed a small, dense, granular and slightly irregular R type of colony and a less granular, round, entire S variant, which was composed of longer and thinner organisms than the former. In the course of dissociation the variants passed through a stage in which the colony was large, irregular and granular. Cowan's observations indicated that the virulent form dissociated to a non-virulent and atoxic type, but she did not find the R organisms or filtrates of these organisms of use as antigens to produce immunity against the virulent organisms of the same strain. Her observations on virulence are not in agreement with those made by Slawyk and Manicatide in 1898 who found that the irregular, matt colonies killed guinea-pigs more rapidly than round, glistening forms.

Recently two contributions to the study of the diphtheria bacillus have been made by Y^u (1930) and by Anderson, Happold, McLeod and Thomson (1931). Y^u isolated strains from patients in the active and convalescent stages of the disease and he found that smooth/

smooth, virulent and toxic strains were transformed to non-virulent R forms in the throats of patients during convalescence. In some cases the transformation passed through an intermediate stage in which diminished toxin formation was not associated with morphological change. Examination of colonies was made on blood-agar whereas the work of Anderson, Happold, McLeod and Thomson was carried out on a specially rich medium containing potassium tellurite and rabbit's blood.

Under such conditions these authors claimed that it was possible to differentiate the diphtheria bacillus from other diphtheroids and the medium was, therefore, of practical value for diagnostic purposes. An irregular, lustreless colony which fermented polysaccharides and grew in bouillon with a granular deposit and pellicle was associated with severe toxic cases, while a convex, glistening form which did not ferment polysaccharides and grew in bouillon in a uniform turbidity was associated with milder cases. Some intermediate types were observed.

From this brief summary it may be seen that the question of variation in the diphtheria bacillus and allied forms presents a problem of great practical importance.

The Diphtheria Bacillus.Biochemical Reactions and Virulence.

Forty-four strains of the diphtheria bacillus were isolated from cases in the active stage of the disease. Forty of these fermented dextrin, galactose, glucose and maltose but not lactose, mannite or saccharose. Two strains failed to ferment dextrin when first isolated but when examined after several months both produced acid from this carbohydrate. One strain fermented saccharose but when retested later had lost this property. These three strains were all virulent. Four of the remaining forty-one strains showed negative reactions when tested for virulence; the remainder were positive.

Twenty-four strains were isolated from cases in the convalescent stage of the disease. Fifteen of these gave "typical" fermentation reactions and were virulent, while six were avirulent. Two other strains were virulent but one fermented saccharose and the other failed to ferment dextrin. (The stability of these reactions was not examined as the strains were not maintained in culture.) One other strain was found to ferment saccharose and it also showed a weak-ly/

weakly positive reaction when tested for virulence.

Two strains isolated from carriers were examined and both were virulent and gave characteristic fermentation reactions.

Colony Structure of Recently Isolated Strains on Loeffler's Serum and Tellurite-Agar.

When the foregoing strains were examined on Loeffler's serum no colony was observed to which the term "rough" could accurately be applied. There were slight differences in the surface appearance of the colonies, some being more glistening than others, but the general structure was round and smooth. On tellurite-agar the form was similar but in three strains the colonies were irregular in outline; on Loeffler's serum they presented a smooth entire appearance and when subcultured after some weeks onto tellurite-agar they reverted to a "smooth" form.

Colony Structure on Loeffler's Serum, Blood-Agar, and Agar after Subcultivation on Loeffler's Serum Slopes.

Several months after isolation twenty-six of the strains isolated from patients in the active stage of the disease and sixteen from patients in the convalescent stage were reexamined on Loeffler's serum. They were/

were also plated on blood-agar and on ordinary nutrient agar. On the first two media no "rough" colonies were observed. On agar marked differences in the structure of the colonies were noted. S colonies only were found in five of the strains from active and seven from convalescent cases. The remainder showed matt, granular colonies varying in size and shape from minute, irregular forms to large, spreading or dense, irregular colonies raised in the centre and sloping towards the edge. Six strains isolated within two weeks from active cases were also examined. In three of these S colonies only were found but the colonies of the three other strains were irregular but glistening on serum and blood-agar and on ordinary agar they were flat and granular with either a matt or glistening surface.

Toxin Production.

A number of strains were tested for toxin production. They were grown in Hartley's broth for ten days, filtered and tested by injecting 0.1 c.c. intracutaneously into guinea-pigs. The results of these tests and of the foregoing observations are summarized in the following tables.

Summary/

Summary of Fermentation Reactions, Virulence, Colony Types (when first isolated)
and Toxin Production of Strains of B. diphtheriae.

Active Cases

No. of Strains	Dext- :rin	Galact- :ose	Gluc- :ose	Malt- :ose	Lact- :ose	Mann- :ite	Sacchar- :ose	Virul- :ence	Colonies on		Toxin Production - No. of Strains Tested Toxic Atoxic		
									Tellur- :ite agar	Loeff- :ler's serum			
34	+	+	+	+	-	-	-	+	S	S	9	7	*2
1	+	+	+	+	-	-	-	+	R	S	1	1	0
3	+	+	+	+	-	-	-	-	S	S	2	2	0
2	(+)	+	+	+	-	-	-	+	S	S	2	2	0
1	+	+	+	+	-	-	(+)	+	S	S	1	1	0

Convalescent/

* It should be remarked that by the method employed 4 strains showing the morphological characters and biochemical reactions of a diphtheria bacillus were atoxic. It is possible, however, that by injection of more than 0.1 c.c. of filtrate (the amount used in these tests) a positive reaction would have resulted. (See table on following page.)

Convalescent Cases

No. of Strains	Dext-:rin	Galact-:ose	Gluc-:ose	Malt-:ose	Lact-:ose	Mann-:ite	Sacchar-:ose	Virul-:ence	Colonies on		Toxin Production		
									Tellur-:ite agar	Loeff-:ler's serum	Tested	No. of Strains	Tested
14	+	+	+	+	-	-	-	+	S	S	2	2	0
1	+	+	+	+	-	-	-	+	R	S	1	1	0
5	+	+	+	+	-	-	-	-	S	S	2	1	0
1	+	+	+	+	-	-	-	-	R	S	1	0	*1
2	+	+	+	+	-	-	+	+	S	S	0	0	0
1	-	+	+	+	-	-	-	+	S	S	0	0	0

Carriers

No. of Strains	Dext-:rin	Galact-:ose	Gluc-:ose	Malt-:ose	Lact-:ose	Mann-:ite	Sacchar-:ose	Virul-:ence	Colonies on		Toxin Production		
									Tellur-:ite agar	Loeff-:ler's serum	Tested	No. of Strains	Tested
2	+	+	+	+	-	-	-	+	S	S	1	0	*1

Summary of Colony Types on Loeffler's Serum,
Blood-agar and Nutrient Agar.

Number of Strains from <u>Active Cases</u> .	Colonies on		
	Loeffler's Serum	Blood-agar	Agar
5 (cultivated on serum for sev-	S	S	S
21 (veral months.	S	S	irregular in form, matt or glistening.
3 (cultivated on serum for not	S	S	S
3 (more than two weeks.	irregular, glistening.	irregular, glistening.	irregular, glistening, or matt.
Number of Strains from <u>Convalescent Cases</u> .	Colonies on		
	Loeffler's Serum.	Blood-agar	Agar
7 (cultivated on serum for sev-	S	S	S
9 (veral months.	S	S	irregular, glistening, or matt.

From/

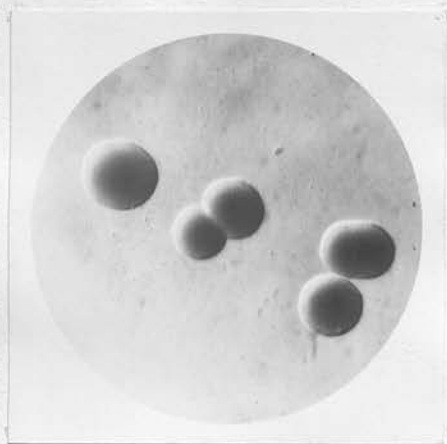
THE DIPHTHERIA BACILLUS

Fig. 94. Variant I.
2 days' growth - agar. x 20.

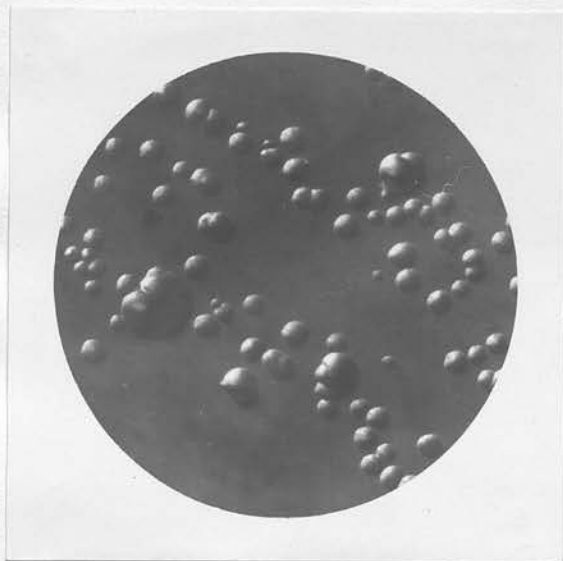


Fig. 95. Variant I.
2 days' growth - agar. x 20.

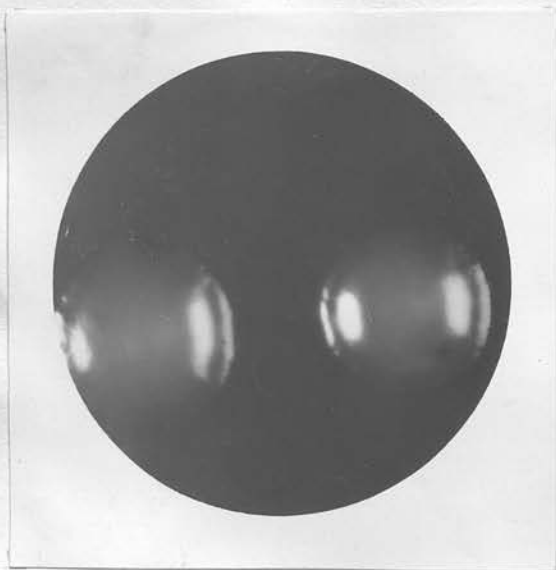


Fig. 96. Variant I.
2 days' growth - blood-agar.
x 20.

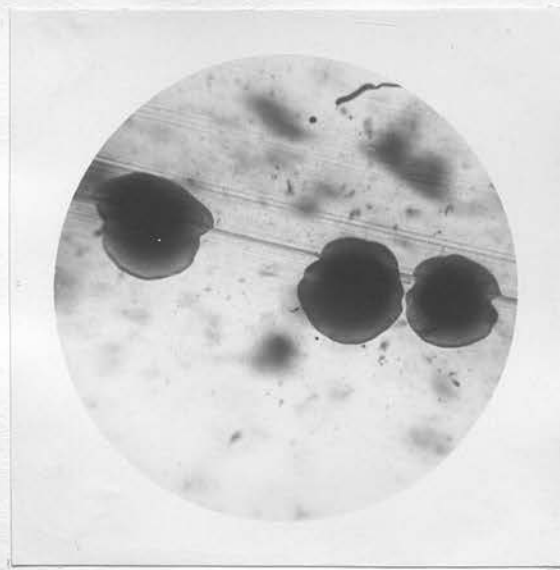


Fig. 97. Variant I (atypical)
2 days' growth - agar. x 20.

From the foregoing tables it appears that in certain strains, the medium on which the organism is grown influences to a considerable extent the type of colony which develops. It is also of interest to note that a greater percentage of avirulent strains was isolated from convalescent cases than from cases in the early stages of the disease.

Dissociation Experiments.

Three strains of the diphtheria bacillus were transferred alternately from agar to bouillon at intervals of two days, for a period of several months. Colonies showing "smooth" and "rough" characteristics were selected from each strain. It was found, as in the case of acid-fast organisms, that there was a considerable difference in the colonies of these strains of Corynebacteria, and that the types were relatively unstable. Some of the variants which have been observed are described below:

Variant I. This was a round, smooth, glistening colony, easily emulsified in saline and corresponding to the usual descriptions of "smooth" variants. There was considerable difference in the size of these colonies not only in the different strains but also in the same strain and on one medium (Figs. 94 to 96). A closely related form was characterized/

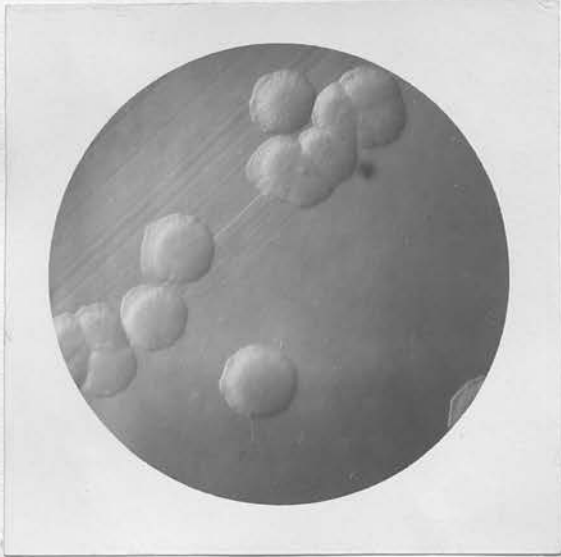
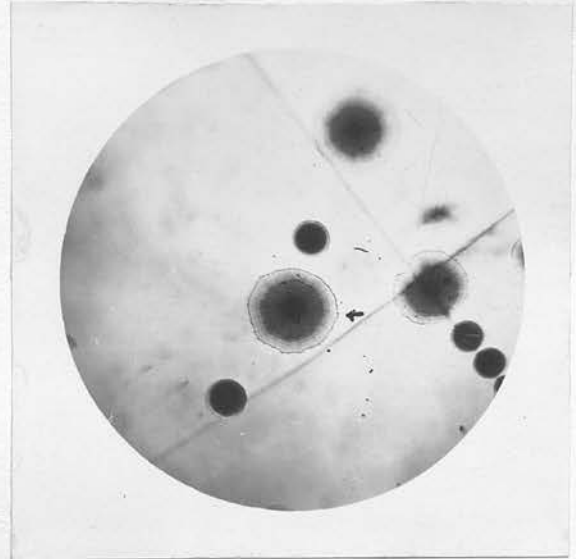
THE DIPHTHERIA BACILLUS

Fig. 98. Variant III.
2 days' growth - agar. x 20.



IV

Fig. 99. Variant IV.
4 days' growth - agar. x 10.



Fig. 100. Variant V.
2 days' growth - agar. x 20.



Fig. 101. Variant V (atypical)
2 days' growth - agar. x 20.

characterized by a central dome and thick, entire periphery. The surface was smooth or granular and usually glistening. A somewhat similar but less regular type is shown in Fig. 97.

Variant II. This colony was convex, opaque and dense. The edge was irregular or "nibbled" and the surface matt (not illustrated).

Variant III was flat and matt with a granular surface and somewhat irregular edge. It was difficult to emulsify in saline. In some colonies the edge was entire (Fig. 98).

Variant IV. This was flat, thin and veil-like. Sometimes it was slightly raised in the centre or at the edge. It was difficult to emulsify (Fig. 99).

Variant V: was extremely small and rough with a crenated edge (Fig. 100). In other colonies of a somewhat similar structure the edge was more regular (Fig. 101). The organisms of which these colonies were composed did not differ from those of larger colonies.

Variant VI. This colony was large, spreading and granular. It was raised in the centre and sloped down to a thin, matt periphery (not illustrated).

These more outstanding types were observed and also less characteristic forms, for example the flat, granular colonies shown in Figs. 102 and 103 and colonies combining both glistening and matt growth (Fig. 104). The extremely minute forms shown in Fig. 105 were not, as far as could be ascertained, identical with Variant V, and were as a rule associated with a series/

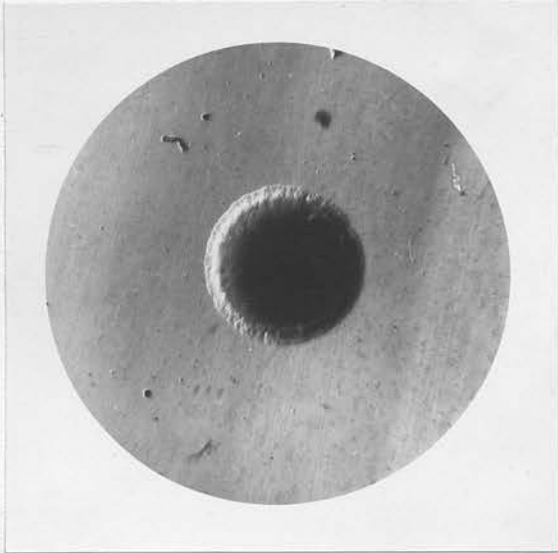
THE DIPHTHERIA BACILLUS

Fig. 102. Granular Variant.
2 days' growth - agar. x 20.

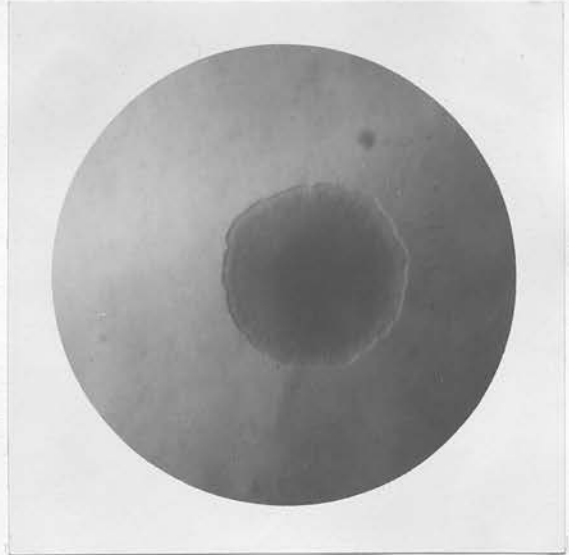


Fig. 103. Granular Variant.
2 days' growth - agar. x 20.

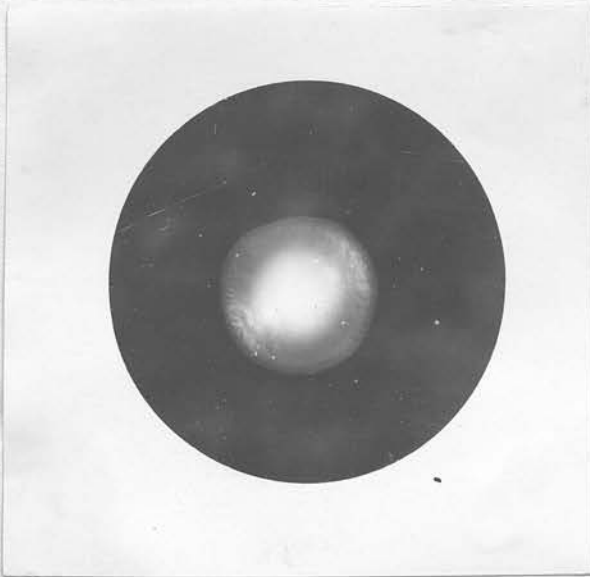


Fig. 104. Glistening colony
with matt edge - agar.
2 days' growth. x 10.

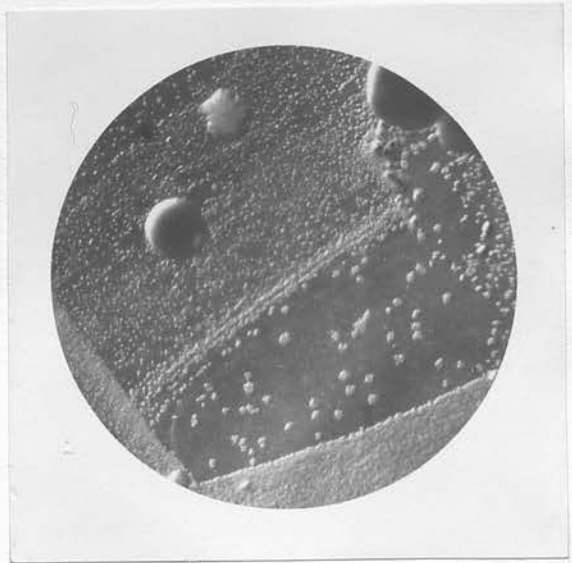


Fig. 105. Three different
colony forms - agar.
2 days' growth. x 20.

series of types varying between the extremely small forms and one or other of the larger types. In several instances the small irregular colonies were obtained by plating segments of fine growth such as that which composed the colonies in Fig. 99 but they also arose from round, opaque, matt colonies.

Matt and glossy variants of several strains were tested for virulence and toxin production but no marked differences between the types were observed.

Interchangeability of Types.

In two of the three strains examined in detail all of the various colonies described were observed. The types were not stable and changed from one form to another, without any obvious sequence. In the third strain small colonies were not observed.

Papillae and Segment Formation.

The small rough variants (Fig. 101) frequently formed papillae from which smooth colonies could be derived, and supergrowths were also observed on other types, especially if the colonies were left at room temperature for a day or two. Segments of growth differing from that of the parent colony were frequent in both/

both glistening and matt colonies.

Growth in Fluid Medium.

In fluid medium the types of growth varied from a uniform turbidity to a clear fluid with a few granules at the bottom of the tube. The turbidity was sometimes of a finely granular appearance and in such a case the sediment was granular rather than compact. Surface growth was present either on a turbid or a clear fluid and varied in thickness. The different types of growth have all been observed in one strain and while smooth glistening forms were frequently associated with turbid growth this was not always the case. For example, glistening smooth colonies were derived from a clear bouillon culture with granular deposit; from another culture of the same strain and similar in appearance, forms varying from minute irregular types to thick round matt variants were observed. Matt and small colonies have also been obtained from turbid cultures of the same strain.

In general a more turbid type of growth seemed to be associated with glistening colonies and other closely related types, but it was not found possible to predict the type of colony that would arise from a certain type/

type of growth in bouillon and conversely, by subculturing a particular colony in bouillon it was not always possible to tell what type of growth would result in fluid medium.

Dissociation Induced by Growth in Anti-bacterial Serum.

Several attempts were made to induce dissociation by growth in diphtheria anti-bacterial serum. In one instance two strains were cultivated in 10 per cent. antiserum bouillon, subcultures being made on Loeffler's serum. After the fifth transfer at twenty-four hour intervals both strains formed matt colonies raised in the centre and sloping to an irregular edge. The surface was rough. The homologous strain reverted to the smooth form when replated on serum and also after further transfers in bouillon. The heterologous strain formed rough colonies when plated after one more transfer in bouillon but the types were not observed again, and, owing to lack of serum the experiment had to be discontinued after five more transfers. This is the only occasion in which a "rough" colony from a "typical" strain of B. diphtheriae was observed on Loeffler's serum. Other attempts to produce similar forms were unsuccessful.

Other/

Other Diphtheroid Bacilli.

Nine other strains of diphtheroids were isolated from the throats of convalescent diphtheria patients. All were avirulent and their colonies on serum were irregular, spreading and rough and on agar and blood-agar were flat, matt and irregular. Three strains which fermented galactose, glucose, maltose and saccharose but not dextrin, lactose or mannite, were apparently similar to B. xerosis. The other six strains fermented dextrin, galactose, glucose, maltose and saccharose but not lactose and mannite.

The organisms of these six strains were diphtheroid in shape and were gram-positive. Granules were present in some cells but were not uniform throughout the culture. Smears made from old cultures of two strains showed large, gram-negative, globular-looking cells. The growth from which these smears were made appeared to be the same as that of the culture when first isolated. When subcultured and reexamined the organisms were found to have become gram-positive and diphtheroid in form. One strain of the six showed two types of colonies on serum, the one irregular, somewhat raised and glistening and the other large, flat, spreading and matt. There was no apparent difference in the cells composing these colonies.

Summary of Observations.

In recently isolated strains of the diphtheria bacillus colonies of a smooth structure were found to be associated with both active and convalescent cases, and "rough" forms such as those described by Y^d were not observed on blood-agar or on Loeffler's serum. Marked differences in the colony structure occurred on agar, but these disappeared on serum, a fact noticed by Bernhardt in 1915. Apparently, however, rough forms do occur on this medium, as for example after growth in bouillon containing antiserum. This transformation was only observed in two strains and was not permanent.

The strains of diphtheroids which presented a rough colony structure and fermentation reaction differing from those of the diphtheria bacillus and which were isolated from convalescent cases were not found to undergo a change to forms similar to the true diphtheria bacillus.

It is of particular interest to note that two of the three strains of B. diphtheriae examined in detail occurred in six different colony forms and several atypical varieties, none of which were stable. Growth in/

in bouillon also showed variation. In the work of Yd (1930) and of Anderson, Happold, McLeod and Thomson (1931) a sharp classification of colonies into two distinct forms and intermediate types (in a few strains) is made, but from the observations recorded here, although limited in scope, the question is raised as to whether bacterial growth is sufficiently stable to justify a classification based mainly on colony structure and growth in bouillon.

Yd found the virulent and toxic S variant was relatively stable on blood-agar but could be transformed to an R form by cultivation in anti-bacterial serum. He did not succeed in causing reversion of the R variant by the use of similar methods. The irregular daisy-head and highly virulent colony and less virulent, smooth glistening form described by Anderson and his co-workers, are reported as stable after cultivation under artificial conditions for periods varying from six weeks to eight months. The form of colony, growth in bouillon and fermentation reactions were unaltered when retested and the two variants were not found to be interchangeable after growing on agar at 37°C. or standing in diffuse light for some weeks at room temperature. It would be of interest if a comparison could/

could be drawn between the work of the investigators mentioned above and the results recorded in this paper, but as the media used for the investigations were different this is not possible. It would appear, however, that blood-agar and chocolate-tellurite medium exert a stabilizing influence on variants of B. diphtheriae. Agar, on the other hand, although differentiating a greater number of types fails to stabilize them. At present there is no explanation for the fact that in the strains studied by Anderson, Happold, McLeod and Thomson the two types of growth in bouillon were not observed to undergo variation whereas the organisms investigated in this study showed considerable variability when cultivated in fluid medium.

This work has shown how B. diphtheriae under certain conditions may assume a variety of colony forms, although these do not constitute stable variants. This is of special interest at the present time in view of the recent claims by Anderson, Happold, McLeod and Thomson (1931) that types of B. diphtheriae can be differentiated according to colony characters and growth in bouillon, etc., and that these differ in virulence and toxicity. These workers have formulated a classification/

classification of strains of B. diphtheriae based on their findings but the question might be raised, in view of the observations recorded above, whether such an exact classification is justifiable. The question obviously requires much further and detailed study but the data given above are sufficient to indicate the great variability and instability of the diphtheria bacillus in its colony characters under certain environmental conditions. The classification as suggested by Anderson, Happold, McLeod and Thomson must therefore be accepted with reservations.

DISCUSSION AND CONCLUSIONS

A study has been made of dissociation in strains of a virulent bovine, attenuated bovine (B.C.G.) and a human tubercle bacillus, and of the more rapidly growing fish tubercle and "leprosy" bacillus. A less detailed examination has been made of the tortoise tubercle bacillus and Mycobacterium rubrum. A number of strains of diphtheroids have also been examined. The most outstanding colony types have been described and an examination made of their stability and of some related characters such as virulence (in certain strains), emulsibility in saline and changes in cell morphology. The variants have been numbered in preference to classifying them as S, R and I forms; it seemed desirable to investigate the intermediate types in some detail, as early in the work it became evident that among acid-fast organisms such forms were more numerous than one was led to expect from the general literature on microbic dissociation among either rapidly or slowly growing organisms. Each variant described was definite in its characteristics and was clearly differentiated from other colonies of the same species/

species.

Since Arkwright (1920) first suggested that the symbols S and R be used to designate colony variants of microorganisms and since Hadley (1927) reviewed the literature on the subject and attempted to demonstrate that a certain trend of dissociation was common to all bacterial species increasing emphasis has been placed on those two colony types to which the symbols were applicable. Such a systematic classification naturally arose when it was found that certain variants maintained their characteristic form in subsequent generations, and as a result of this it became essential to have available suitable terms of reference for dissociated cultures of bacteria. The resulting effect upon literature dealing with variation of colony structure and other associated characters has been to emphasize these so-called S and R variants, and while such emphasis may be justified up to a point it is certainly not justified in stressing these two forms out of all proportion to the other variants occurring within the species. Unfortunately this seems to be the case in the majority of publications dealing with dissociation, for, while many investigators report that one or more intermediate forms have been observed, only a few have studied in detail/

detail the trend of dissociation among such forms. At the present time it is generally recognized that the trend of dissociation, in most bacterial species, occurs from S \rightarrow R, with or without the appearance of intermediate colonies and that the R variant exhibits a greater degree of stability than the S, and both are more stable than the intermediate types. Various investigators such as Löhnis and Smith (1923) working with variants of Azotobacter and Hadley (1927) with R cultures of B. pyocyaneus and Friedländer's bacillus found that the variants remained constant over a period of years. Gratia (1921) reported that S and R forms of B. coli remained true to type after passage through experimental animals. Similar observations were made by Baerthlein and Toyoda (1913) in the case of variants of the frog tubercle bacillus. Amoss (1925) regarded the stability of the R form of the streptococcus as indicative of a true mutation from the S. Similar results have not been obtained in all species. Arkwright (1920) observed that the S and R colonies of organisms of the typhoid-dysentery group were stable when subcultured in bouillon at intervals of one week, but more frequent subcultivation favoured the former and prolonged periods the latter. Other investigators have/

have reported instability in the two main types of colonies and Tzeknovitzer (1930) working with human, bovine and attenuated bovine (B.C.G.) tubercle bacilli and with the timothy grass and smegma bacilli observed that on subcultivation not only did the S and R colonies lose the characters they first presented but the types were also reversible. Variants of the rat "leprosy" bacillus were also found to be interchangeable (Kahn and Schwartzkopf, 1932). Soule (1928), Eagles (1928) and others have found it possible to stabilize colony types by prolonged selective subcultivation but since it has frequently been shown that variants can be made to dissociate when subjected to a suitable stimulus it seems doubtful whether the characters stabilized in this way would persist under altered conditions. Among slowly growing acid-fast organisms it has only been possible to make a limited number of subcultures but so far the results correspond to those of Tzeknovitzer since variants which were derived from cultures of the human and bovine tubercle bacillus and from a strain of B.C.G. have exhibited no absolute stability. Similar observations have been made in the case of the fish tubercle bacillus, the "leprosy"/

"leprosy" bacillus and certain strains of B. diphtheriae. It has been remarked that no absolute stability has been recorded but it must be pointed out that subcultures of certain variants showing the generally recognized characters of S and R forms have resulted in a preponderance of types similar to the parent colony, the remaining forms being atypical or of a totally different structure. Most of the strains showing dissociation had been cultivated in artificial conditions for some time and were therefore representative of the average stock culture of acid-fast bacilli and of the reactions taking place in such a culture.

In this study of acid-fast and diphtheroid organisms the outstanding feature of the results has been the wide variety of different colony forms observed in the various species examined. There is, however, a considerable difference between the complex colonies of slowly growing Mycobacteria and the relatively simple colonies of the majority of more rapidly growing organisms, and at first it would almost appear as though the number of variants increased in direct proportion to the primary complexity of the colony structure. A further consideration of the literature, however, shows that/

that, while some investigators refer to S and R forms only, the majority give some indication of the existence of other forms, and in the literature prior to 1921 when Arkwright's work on dissociation was published Eisenberg, Baerthlein and various others reported variation in many species and recorded a number of colony types in each of the different strains examined. But supporters of a systematic classification of variants pointed out that the symbols S and R were applicable to certain of the colonies described by these earlier workers and Hadley in emphasizing the sequence from "smooth" to "rough" believed that the importance of the two variants had previously been "overshadowed by a wealth of detail, often including some inconsequential aspects of dissociation". It is, however, to such work one must refer if one wishes to obtain a true idea of the changes taking place in cultures of microorganisms, since the more recent literature, with but few exceptions, fails to contribute to the study of the "intermediate" forms. These intermediate variants are frequently found to be unstable and are therefore unsatisfactory types with which to work but they do occur in many, possibly in all, bacterial species and therefore/

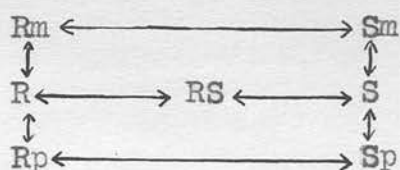
therefore should receive a more detailed study for the better understanding of the changes taking place in microorganisms both in vitro and in vivo. Among both acid-fast and diphtheroid organisms many of the variants intermediate between S and R were definite in their characters and while the change from one form to another was frequently abrupt there were also observed colonies of indefinite structure combining the characters of more clearly defined forms. From time to time it was noticed that when several plates were seeded with the same inoculum one developed one type of colony while a second developed quite a different type. On a third plate both variants might be present. At first it seemed possible that differences in environmental conditions might be responsible for this variation but as far as could be ascertained there were no obvious differences in the physical state of the medium in the various plates, and it had all been prepared in one batch. So far an explanation of this peculiarity has not been forthcoming and it is regarded as a matter which requires further investigation.

It was found that while variation occurred readily on one medium alteration in the appearance of a colony was/

was due, to a considerable extent, to alteration in environmental conditions, the structure of the colony and, in some cases, the number of variants depending on the medium on which the organism was subcultured, as for instance when the rough Variant II of the "leprosy" bacillus was transferred from gentian-violet-egg to blood-agar medium where Variants V and VII and sometimes VI developed. These variants were of a somewhat different structure than Variant II and yet reverted to that form when replated on gentian-violet-egg medium. Variant I (the S form) of the same strain retained its smooth glistening appearance on blood-agar. On Loeffler's medium Variant II reverted more rapidly to Variant I than on any other media. Presumably on certain media some cells underwent dissociation more readily than others.

Among the diphtheroids and the more rapidly growing acid-fast organisms a trend of dissociation from S \rightarrow R was observed. The reverse reaction also took place and the different intermediate forms could be transformed from one type to another. In the fish tubercle bacillus Variant VII (Fig. 76, page 149) was obtained by plating an unusually wide border which had developed/

developed on Variant IV. This colony did not appear to have any place in the sequence of variants ranging between S and R and it seemed possible that variation besides occurring in a line between two distinct types also branched off in some other direction. Insufficient work has been done on this subject with acid-fast organisms but the possibility is suggested by the work of Nungester (1929) who investigated B. anthracis and found that the S → R variation only represented one phase in a more complex scheme. The main variants which were observed he designated "rough" (R) and "smooth" (S) but he also observed colonies of a mucoid (m) consistency and a thin, veil-like phantom (p) growth and a combination of these characters and the trend of dissociation he represented thus:



In vivo the trend of dissociation was similar to that observed in vitro and only part of this scheme conforms to the S — I — R sequence of dissociation.

Among acid-fast organisms other types of variation occurred/

occurred, which, unlike emulsibility in saline and growth in bouillon, were not associated particularly with any of the colony types. Variants of the "leprosy" bacillus and Mycobacterium rubrum showed increased pigment production although developing colonies similar in form to the less pigmented strains. Reed and Rice (1929) reported that yellow, brown and red pigment production among acid-fast organisms was related to the presence of iron in the medium and therefore variation in colour in the two strains examined must have been due to the ability of some of the organisms to utilize the iron to a greater extent than other cells, since both types of variants were observed on one plate. Similarly with the fish tubercle bacillus some cells possessed the power of taking up the dye from gentian-violet-egg medium while others lacked this power. The general structure of the colonies was somewhat similar in both cases.

It has frequently been emphasized that in pathogenic species, with the possible exception of the anthrax bacillus, S variants possess a higher degree of virulence than R forms of the same species. Petroff found this to be the case with the strains of bovine and attenuated/

attenuated bovine (B.C.G.) tubercle bacillus which he examined. Other investigators have confirmed these findings working with variants of B.C.G. obtained from Petroff but have failed to confirm them with variants derived from cultures dissociated in their own laboratories. Begbie has shown that an even higher degree of virulence was associated with a colony type differing somewhat from that described as S by Petroff, while in the results recorded in this paper neither the variants similar to those described as virulent by Petroff or by Begbie produced extensive tuberculous lesions, but the S variants when injected into guinea-pigs exhibited a slightly greater degree of virulence than the other types. It seems evident that while colony appearance may be an indication of virulence in one strain it is not necessarily so in all strains of the same species. Similar observations were made by Eagles for haemolytic streptococci since in one strain a "smooth" form might be the more virulent while in another it was the "rough", and Tzeknovitzer reported that variants of a virulent strain of tubercle bacillus were virulent while those of an avirulent strain were avirulent. Rabbits and guinea-pigs injected with "smooth"/

"smooth" and "rough" variants of the bovine tubercle bacillus and guinea-pigs injected with similar types of B.C.G. did not show any marked differences in virulence.

The question arises "What underlies the phenomenon of dissociation?" In the foregoing study it has been amply demonstrated that while environmental conditions may influence the occurrence and the rate of dissociation they are not solely responsible for it. If the changes occurring among bacteria were confined purely to colony variation they would not be of great importance but when they are correlated (sometimes, but not always) with changes in antigenic structure, virulence and biochemical reactions, to mention some of the more important characters, they assume a greater importance especially when it is necessary to keep cultures in stock for a considerable period for use in diagnostic tests or for other purposes. Hadley has suggested that the R variant is the completely or partially stabilized form resulting from the germination of special cell structures, such as zygospores, and that the S types surviving dissociation are the remnants of the original culture which have not entered upon the modified/

modified reproductive process but have persisted in a limited vegetative reproduction of the same form. To prevent dissociation conditions leading to the formation of the special cell structures must be eliminated and to stimulate dissociation those conditions favouring their production must be brought about. This theory is the most comprehensive one put forward in explanation of dissociation so far, but insufficient examinations of changes in cell morphology have been made to add any constructive contribution to it. Since, however, it seems evident that an explanation of dissociation is not to be found in a study of colony variants alone, a more detailed examination of variation occurring among individual organisms might throw more light on the subject and possibly bring forward evidence which would support Hadley's hypothesis.

From the foregoing observations it seems evident that the number and appearance of colony variants, their associated characters and their rate of dissociation depend to some extent, but not absolutely, upon factors such as the colony selected for investigation, the environment, the rate of subcultivation and the age of the colony when subcultured. It seems highly doubtful whether such a thing as "absolute stability" exists among/

among the various characters of microorganisms.

It may be suggested that all these variations in colony appearance are artificial, dependent perhaps on the environmental conditions to which the colony is subjected from the time it first begins to develop, or that the colony form is haphazard and not a true "variation".

Undoubtedly in certain cases modifications in colony structure appeared to be due entirely to the influence of environment. For example, ageing and consequent dehydration resulted in changes in the shape and surface of colonies (bovine tubercle bacillus, Figs. 1, 2 and 4, page 52; Fig. 5, page 54). As was to be expected, however, the general appearance of the colonies was not entirely changed. Variant V (Fig. 58, page 125 and Fig. 62, page 127) and Variant VII (Figs. 60 and 62, page 127) of "B. leprae" on heated-blood-agar were of a finer structure than the same forms when grown on blood-agar. When transferred from the latter medium to the former a change to the finer type occurred and when these were again subcultured on blood-agar the structure became coarser. It was observed that modifications such as these, depending on environmental conditions, occurred in other species (fish tubercle/

tubercle bacillus, B. diphtheriae, etc.). Not only were the colonies affected but in the case of the "leprosy" bacillus the organisms when grown on gentian-violet-egg medium, bouillon or Loeffler's serum were considerably larger than those on glycerine-egg or agar media.

It was also observed, however, that in every species investigated, either on gentian-violet-egg medium or on some other medium, more than two colony types occurred. In appearance these were not simply modifications such as those described above, but they differed in a marked degree from each other, not only in shape but also, frequently, in associated characters such as the size and arrangement of cells composing the colonies, the ease with which the growth was emulsified in physiological salt solution, and the antigenic structure. It is to forms showing these marked changes that the term "variant" is applied. Some of the more striking examples of colonies of different structure in the same species may be seen in variants of the bovine tubercle bacillus (Fig. 1, page 52; Fig. 10, page 56; Fig. 15, page 58), B.C.G. (Figs. 16 and 17, page 78) and "B. leprae" (Figs. 50 and 51, page 120). The variants were unstable and frequently interchangeable, although/

although subcultured either on media made in different batches or on media made up in sufficient quantities to last throughout the entire experiment and which may, therefore, be regarded as relatively uniform. When a particular colony type was selected and plated by the "single-cell" filtration technique advocated by Petroff there might be no change in the resulting colonies or forms quite different from the original might be thrown off even though selective subcultivation was carried out for a prolonged period. There are several other aspects of dissociation which suggest that environment alone is not responsible for the phenomenon.

Most of the species examined showed the development of papillae or of segments of growth differing from the parent type. In "B. leprae", B. diphtheriae and the fish tubercle bacillus such segments were usually more difficult to emulsify than the smooth parent colony, and in the case of the latter organism the new type of growth also differed from the original in being unable to take up the dye from the gentian-violet-egg medium. In the bovine tubercle bacillus shorter cells were usually associated with "rough" colonies and this was also the case with "B. leprae", irrespective of the medium on which it was cultivated.

The/

The changes were not gradual suggesting that environmental conditions were slowly causing an alteration of form, but they occurred abruptly in young as well as in old colonies. In the case of pigment variation in Mycobacterium rubrum and the "leprosy" bacillus a segment differing from the parent type could be detected early in the development of a colony and, as a rule, such a change only occurred in one, or at most a few, but never in all the colonies on a plate. As a colony aged it frequently developed a border of another type of growth as in the case of B.C.G. (Figs. 31 and 32, page 85), "B. leprae" (Fig. 54, page 122) and the fish tubercle bacillus (Fig. 78, page 149). If dissociation were due solely to environmental conditions one would expect different variants to develop in different conditions. This, however, is not altogether the case and one type of growth may occur under the most diverse conditions. For example, a particular variant might occur on moist fresh medium either as a colony, or as a segment; it might appear as the periphery of colony on a medium which has become dry or it might be derived from growth in fluid medium inoculated with the parent type. It seemed probable, therefore, that some factor other than environment was responsible, in/

in part at least, for causing, under such varied conditions, not just a modification but a definite change to a type of growth which was the same in each case and which differed widely from that of the parent colony. Assuming, for the moment, that environment was responsible for the change one would expect identical cells to react in the same way, providing their surroundings were uniform. It is of course impossible to determine whether this is the case, but it may be assumed that conditions in a Petri dish containing a fairly deep layer of medium are relatively uniform. But, since variants were observed under apparently identical conditions on the same plate, and since changes were found in a part of a single colony (papillae, segments) it was obvious that some cells differed in behaviour from other cells in the same colony and the question therefore arose "Why are certain cells more resistant or susceptible than others to altered conditions?" or rather, "Why do certain cells react in a manner other than that which characterizes the remainder of the organisms in the colony?" The answer to such a question would no doubt solve the problem of microbial dissociation. It has not been found in this investigation.

It cannot be said absolutely that variation is haphazard/

haphazard. In the literature on dissociation it is emphasized that the reaction, with few exceptions, occurs from S to R with or without the presence of intermediate types. The results recorded here point to the fact that while there was a general trend from S to R the change was not a simple one but was complicated by numerous intermediate forms differing either slightly or in a marked degree from the two extreme types and varying in their degree of stability. Besides these were other forms which did not appear to have any place in the sequence of types. It must be emphasized that the trend from S to R did not involve a gradual "roughening" of the colony with every additional subculture but the changes took place abruptly, one variant throwing off a different type of growth. It was not possible to predict when this change would occur. Although it has been stated that variants were unstable and did not dissociate according to any obvious rule it is believed, nevertheless, that the trend of dissociation was from smooth to rough and that the rough forms were not stable but might revert to smooth types. The reasons for this belief are that rough forms which were not observed at first occurred after prolonged subcultivation. (It is not argued that/

that this change does not occur in vivo.) This is exemplified in the appearance of small, crenated colonies in strains of B. diphtheriae (Fig. 100, page 176) which did not originally show such forms. Similarly, in a freshly isolated strain of tubercle bacillus, the colonies were smooth but on further subcultivation threw off irregular and rougher types. In the other strains examined it might be pointed out that since they had not been maintained in artificial conditions for some years at least and had probably undergone various changes, they were therefore unsatisfactory types from which to form an opinion. But, in the strains examined in detail it was observed that colonies to which the term smooth was applicable formed segments and papillae and underwent dissociation much more readily than colonies to which the term rough was applicable. Reversion of the latter type was induced.

In brief, then, it is believed that while environmental conditions modify colony structure to some extent and influence the rate of dissociation they are not solely responsible for the appearance of variants.

The reasons for such a belief are:

(1) Some cells in a colony react in a different manner from other cells of the same colony under apparently/

apparently uniform environmental conditions.

(2) The resulting variant is not simply a modification of the parent type but may differ markedly from it, not only in structure, but also in other associated characters.

(3) The variants are frequently similar in form although developing under entirely different environmental conditions.

(4) Variation is not purely haphazard but shows a certain trend between types which are relatively easily emulsified in saline and, in some cases, are smooth in form, and others which are more irregular and rough in structure and are difficult to emulsify.

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From the results obtained in this study of dissociation among certain strains of Mycobacteria and Corynebacteria it is felt that while the recent literature gives one aspect of the subject in considerable detail (that of the S and R types and their associated characters) it does not present a true picture of the changes taking place when an organism undergoes dissociation. Although the colonies of the two genera examined probably tend to show a greater complexity of form than those of other commonly occurring organisms and/

and consequently allow greater scope for variability, it seems justifiable to assume that similar changes are occurring in other species, although they may be of a less obvious nature. Since, in the majority of cases, the "intermediate types" have received but scant attention from recent investigators (who have come under the influence of one or two systematists) the importance of the reactions among such forms is still unknown. This belief that the changes do occur is strengthened by the reports of early workers who described a considerable number of variants in most of the species examined. The results recorded here are not in agreement with those of many investigators, but they indicate that until a study is undertaken to determine in detail not only the reactions of a few colonies but also those of other forms, there is small probability of understanding the true cause of dissociation.

In the foregoing results it has been shown that to overlook the so-called "intermediate form" as an unstable and unsatisfactory type with which to work, would be to disregard an important aspect of the phenomenon. Reports suggesting hard and fast classifications on the basis of stability of colony structure and/

and certain related characters must be accepted, at present at least, with reservations. The phenomenon, it is believed, is not purely haphazard. It is a definite biological problem of practical importance and as such demands a thorough and detailed investigation in many different species.

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