

G - T Y P E   V A R I A T I O N

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I N   B A C T E R I A

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A   T H E S I S

S U B M I T T E D   F O R   T H E   D E G R E E

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E D I N B U R G H

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April 1937.

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

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The work embodied in this thesis was carried out in the Department of Bacteriology of the University of Edinburgh over a period of four years, during which time the author was successively assistant and lecturer in that Department.

Acknowledgements must be expressed to Professor T.J. Mackie for his interest in the subject; to Professor J.W.S. Blacklock, who provided the strain of bacteriophage used; and to Dr P.B. Hadley for a number of photographs illustrating the cultural features of the G-type colonies in B. dysenteriae Shiga.

Thanks are also due to Miss R. Cleat, late of the Preparation Room of the Department of Bacteriology, for her unfailing attention in all matters concerned with the supply of media for these experiments.

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# G-TYPE VARIATION IN BACTERIA

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G 74/48

G 74/48/15

G 74/30

G 74/30/73

G/158

G 207L

SS2

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P R E F A C E

This thesis reports the results of a series of studies of an organism which the writer regards as a G-type variant, as described by Hadley and others, and includes also a full discussion of the whole problem of the occurrence of this reputed variant. It should perhaps be pointed out that the organism could not be discarded as a mere contaminant of the media employed (e.g., nutrient broth and agar, or MacConkey's medium), since large quantities of the same media, in daily use for other purposes throughout the whole duration of the work, gave no indication whatsoever of such contamination. Further, the presence of the G-variant was in any case by no means constant, and it was frequently absent for long periods at a time.

The experimental part of the thesis has been limited to simple statements of findings and observations. Since the present position of the subject demands the most careful attention and review, it was thought important to include in addition a rather detailed and critical discussion of the whole problem, from the point of view not only of the present work but of the contributions of others.

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REVIEW OF LITERATURE.

Among recent contributions to the study of bacterial variation, perhaps none exceeds the description of the G-variant, by Hadley, Delves and Klimek (1931), in general biological interest. These authors studied in particular a filterable variant of B. dysenteriae Shiga which arose sixteen times during the spontaneous or enforced dissociations of either the S or the R form of the Shiga organism. Hadley regarded this filterable form, distinguished by individual morphological and cultural characters, as gonidial in function, and accordingly referred to it as the G-type.

Confirmatory views were expressed by Hoffstadt and Youmans (1932) in the case of a smooth Staphylococcus aureus freshly recovered from a human infection. During the course of dissociation, enforced by lithium chloride broth and by animal inoculation, SR colonies preceded the appearance of G colonies and frequently showed the development of G colonies at their periphery. The G colony when isolated was similar to Hadley's G type of the Shiga dysentery bacillus in that it was small, translucent, bluish-white, ranging in size from colonies just visible to the naked eye to those barely visible and almost streptococcus-like. On agar slopes it formed

a delicate bluish film of growth. It proved unstable, retaining its G form for from 24 hours to 30 days and invariably reverting to a yellow smooth form. It readily fermented all the sugars fermented by the other forms. Growth occurred more abundantly anaerobically than aerobically. The thermal death point was 75° C. as compared with 55° C. for the normal smooth form, and the G elements were found to pass the densest Berkefeld filter. Morphologically the organisms in the G colonies proved to be Gram-positive cocci about half the size of the normal forms with, in addition, numbers of faintly staining granules. Such a G form was isolated fourteen times, and Hoffstadt and Youmans suggested that the dissociative process in Staphylococcus aureus might proceed in the following manner: SY (whole original organism) -----> SW (smooth white) -----> SR (pseudo-rough) -----> RW (rough white) -----> TG (transitional G) -----> G (gonidial). In subsequent experiments on Staphylococcus aureus, Hoffstadt and Youmans (1934) again obtained forms similar in origin and colony appearance to the G forms of B. dysenteriae, although on this occasion no positive filtration results were obtained (through Chamberland No. 2 filters). It is of interest in this connection that Novak and Henrici (1933), working with aged cultures of a highly pleomorphic Staphylococcus, obtained G-type cultures similar to that described by

Hadley in the dewdrop-like appearance of the colonies on agar plates and their filterability through Berkefeld N and W filters. No reversion was obtained.

In a study of dissociative changes in Eberthella dysenteriae Sonne, Koser and Dienst (1934) observed "small colony" variants similar to the G forms of Hadley and his co-workers. They regarded these slow-growing variants as organisms which had lost the capacity to maintain the normal speed of synthetic activity in building new cell material and whose multiplication rate and colony size were accordingly greatly reduced. This is probably the correct view in their case, but it is doubtful if these authors were justified in regarding their variants as "G-type" in nature inasmuch as the cell elements found in the small colonies did not conform to Hadley's original description. Filtration experiments were uniformly negative, and a gradual reversion occurred towards the normal type. Dienst (1933) had previously described colonies resembling the G type which were found on plating a 30-day culture of B. dysenteriae Sonne in 2 percent. casein-digest medium. The culture was apparently sterile when plated at 2 weeks, but at 3 weeks G colonies, almost invisible to the naked eye, appeared in 24 hours. In this paper

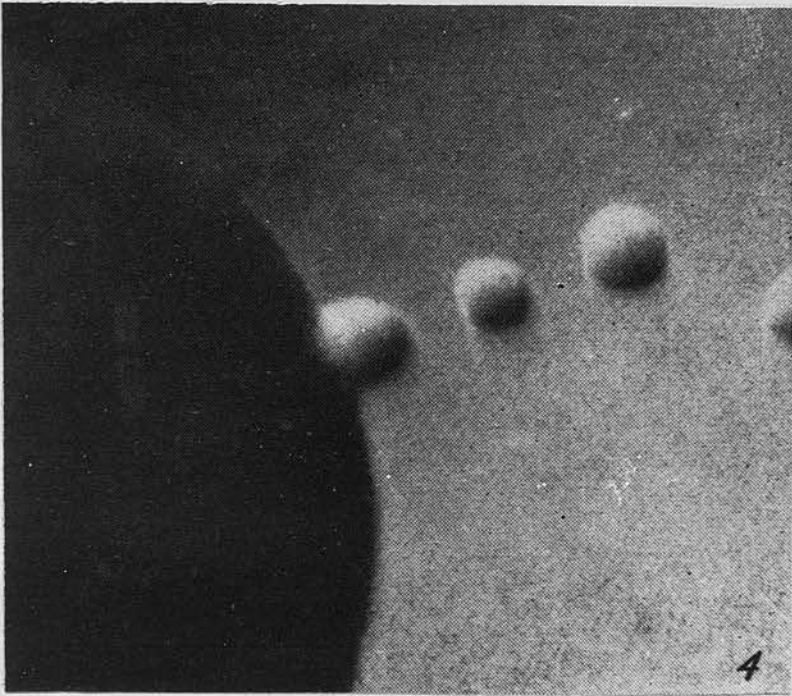


Fig. I. Small colony variants in  
B. dysenteriae Sonne. (Dienst).

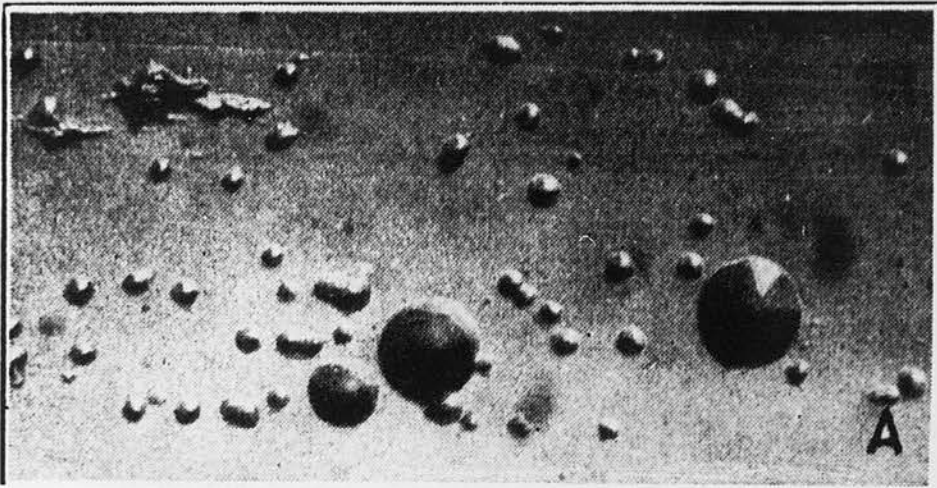


Fig. 2. Small colony variants of  
Eberthella dysenteriae.

(Koser and Dienst)

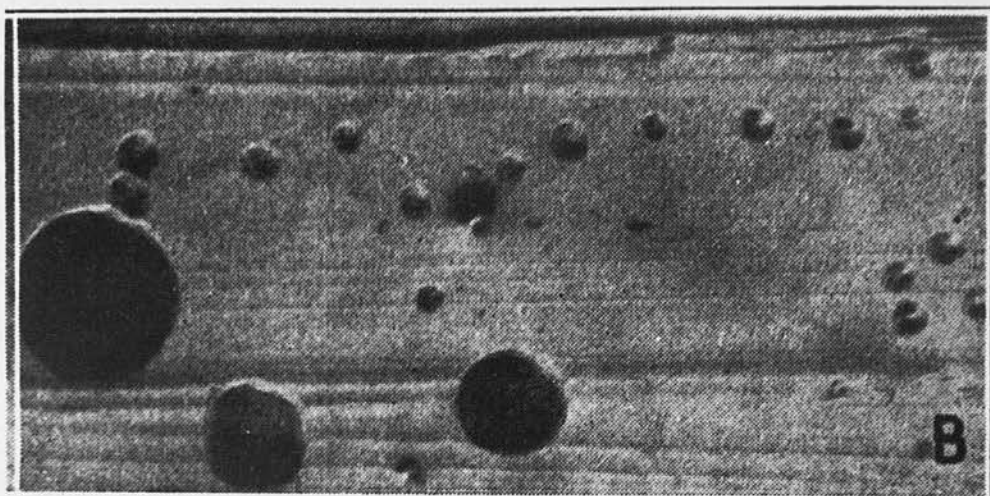


Fig. 3. Small colony variants of  
Eberthella dysenteriae.

(Koser and Dienst).

however Dienst made no reference to the microscopical appearance of such small-colony cultures. Waaler (1935) described similar colonies in one of his dysentery strains, but since these forms were non-filterable, and indistinguishable from the normal either biochemically or serologically, it seems that they represented merely slow-growing modifications, as in the case of the small-colony variants of Koser and Dienst. Figs. 1-3 illustrate the appearance of the variants described by Dienst and by Koser and Dienst.

Fabian and McCullough (1934) described the appearance of G-forms in the dissociation of various yeasts. Cultures from single-cell isolations of Saccharomyces cerevisiae Hansen, Sacch. ellipsoideus Hansen, Willia anomala Saito and Zygosaccharomyces mandshuricus Saito were allowed to age and were subjected to various chemical and physical agencies, including exposure to lithium chloride, brilliant green and ethyl alcohol, desiccation, and incubation at various temperatures. Under these conditions certain definite forms constantly appeared. These were designated as the smooth or S form, the rough or R form, the gonidial or G form and the transitional or T form. The G form consisted of cells greatly reduced in size from that of the S and R forms. They were asporogenic, produced an acid instead of an alcoholic fermentation, and on initial isolation

grew slowly to produce colonies microscopic in size at the end of a week's incubation. The T form was transitional between the S or R and the G form and consisted of a highly refractile organism which produced the G type by the formation of a large number of minute buds on the periphery of the cell. Although fairly stable otherwise, the G organism reverted to the S type on rapid transfer in malt extract broth.

A number of recent observations indicate the wide occurrence of variation of this type, although none of the descriptions agrees entirely with the details given by Hadley as characteristic of the G form. Thus Roe (1934) described the appearance of an avirulent midget type ---resembling the G-type --- in the dissociation of Clostridium welchii. In a study of the dissociation of the gonococcus, Raven (1934) obtained one type of variant growing sparsely and consisting of a fusiform Gram-negative bacillus (2.0 x 2.5/<sup>μ</sup>) and fermenting no sugars. Eaton (1934) described a new pneumococcus variant observed during a series of studies on the S----->R transformation. This variant was characterised by the tendency to undergo a rapid spontaneous lysis under certain cultural conditions at 37° C., and grew much more slowly than the ordinary strains of pneumococcus. It gave rise to "phantom colonies"

which Eaton likened to the G colonies of Hadley. Reversion to normal-growing smooth forms occurred on cultivation in alkaline media and under certain other conditions. Phantom colony variants were also directly isolated from cases of pneumococcal infection in the human.

Swingle (1934) noted the irregular appearance of small colony variants of Staphylococcus aureus on plating ageing broth or agar cultures, and on exposing cultures to lithium chloride in broth. The colony size varied from 0.04 mm. to 0.5 mm., the majority being about 0.1 mm. Morphologically the cellular elements showed no striking difference from the normal but there was evident a greater range in the size of individual organisms. Reversion was obtained on all but three occasions. Originally the staphylococcus attacked dextrose, lactose and sucrose, but the small colony elements fermented only dextrose. The variant cells were characterised essentially by a slow rate of reproduction. The normal strain had only a low degree of virulence to rabbits, but this was quite absent in the small colony type, although most of the variants seemed antigenically similar to the normal. Filtration experiments proved negative. Swingle suggested that the small colony forms occurred as a result of injury to an occasional cell of the normal culture, leading to a lowered rate of reproduction and to the loss of

several physiological properties. In a later paper (1934) Swingle, reviewing the literature, drew attention to the important circumstance that small colony forms are not all strictly alike and may differ in many ways although showing a close resemblance in colony appearance. This observation has been completely substantiated in the course of the present work, and it is of obvious importance in any comparison of different reports in the literature. To mention a concrete example, the small colony variants reported for E. dysenteriae by Koser and Dienst (1934) (see Figs. 2, 3) are almost indistinguishable from G-colonies isolated in the present study (see later figures). But this obvious resemblance is clearly only superficial, since the cellular elements in the former case were bacillary and conformed in no way with the original description for the true G-type colony by Hadley, Delves and Klimek (1931). Several authors have drawn attention to the fact that the so-called dwarf colonies or Zwergkolonien occasionally observed as variants of the Gram-negative intestinal bacteria represent simply slow-growing forms in which the organisms contain a pure O antigen or are deficient in H. Although the colony appearance is similar, such variants have obviously nothing in common with the true G-type, and only confusion can

result if colony appearance is relied on as a sufficient criterion.

In her second paper Swingle gave additional details of the work referred to above. With regard to reversion, only nine small colony variants bred true on repeated subculture over a period of several months, out of several hundred colonies isolated. These variants appeared to have lost the power of pigment production completely. Reversion occurred discontinuously and was looked on as due to changes taking place in an occasional cell but not affecting the culture as a whole. The presence of glucose was found to have a marked effect in inducing the reversion process. In a total of thirty filtration experiments no growth could be detected in any of the media inoculated with filtrates from variant or from normal cultures. No details were given of the precise technique used to determine sterility.

Flynn and Rettger (1934), in a study of variation in B. mesentericus and B. vulgatus, obtained a small colony variant on passage of the original organisms through lithium chloride broth, and small colonies of B. megatherium were obtained by Rettger and Gillespie (1933) by the same method. The small colonies contained bacilli and filtration experiments proved negative. Roos, Reichel and Clark (1934) found similar variants of haemolytic

streptococci which however retained their agglutinogenic and biochemical properties, including toxigenicity but not virulence. Kopeloff (1934) described the appearance of a minute colony occurring in the dissociation of Lactobacillus acidophilus. This conformed closely with Hadley's description of the G colonies, but again filtration experiments were negative.

Chinn (1936 a, b) described the characteristics of small colony variants of Shigella paradysenteriae Sonne and Staphylococcus aureus. He drew attention to (a) the irregular and infrequent occurrence of such forms; (b) their small colony size --- due partly to the greatly increased minimal reproductive time of the variant cells; (c) the greatly reduced or altered biochemical activity, with a reduction of charge and so of cataphoretic velocity. He found the cell morphology to be irregular, and described rod, coccus and filamentous forms. Some strains remained stable, and it was observed that G strains which subsequently reverted showed serological relationship to the normal. The variants with one exception were not filterable through Berkefeld N and W filters or Seitz filters. However, one G colony strain, which did not revert, passed through N filters in about 50 per cent. of tests. Chinn concluded that "while there may be some doubt as to

whether the G-form is really a stage in the life cycle of development there can be no doubt as to its occurrence." In a personal communication to the writer Soule also described the occurrence of phantom colonies, morphologically identical with the G-type, in a large number of bacterial species.

Table I (after Swingle 1935) summarises the main reports of such variant forms in the literature of the last twenty-five years.

T A B L E I

(after Swingle 1935).

Occurrence of small colony variants.

Date	Authority	Organism	Conditions
1910	Jacobsen	<u>Eberthella typhosa</u>	Blood culture.
1911	Fromme	" "	Agar transfers.
1914	Eisenberg	<u>Eberthella typhosa</u>	Old blood-broth and blood-bile cultures.
		<u>Serratia marcescens</u>	Peptone water cultures. Cultures on media containing dyes.
1918	Baerthlein	<u>Eberthella typhosa</u>	Typhoid stools on Conradi-Drigalski med.
		<u>Vibrio cholerae</u>	Cholera stools.
1922	Furth	<u>Salmonella aertrycke</u>	Old broth cultures.
		<u>Salmonella Schottmulleri</u>	" "
1930	Koser	<u>Salmonella aertrycke</u>	" "
1931	Edwards	<u>Shigella equirulis</u>	Direct cultivation and stock cultures.

T A B L E I continued.....

Date	Authority	Organism	Conditions
1931	Hadley	<u>Shigella dysenteriae</u> and other organisms	Serial transfer in lithium chloride broth. Pancreatin broth. Ageing broth cultures.
1931	Kuhn and Sternberg	<u>Escherichia coli</u> (45 strains)	Nutrient broth containing ammonia.
		<u>V. Metchnikovii</u>	Brief exposure to dilute phenol.
		<u>S. aertrycke</u>	
		<u>S. enteritidis</u>	
		<u>S. suispestifer</u>	
		<u>Pasteurella suisseptica</u>	
		<u>Corynebacterium diphtheriae</u>	
		<u>Proteus vulgaris</u>	
		Proteus X-19	
		<u>Shigella dysenteriae</u>	
		<u>E. typhosa</u>	
		<u>Spirillum volutans</u>	
		<u>B. anthracis</u>	
		<u>mycobacterium tuberculosis</u>	

T A B L E I continued.....

Date	Authority	Organism	Conditions
1932	Hoffstadt and Youmans	<u>Staphylococcus aureus</u>	Serial transfers in lithium chloride broth. Intravenous inoculation in rabbits.
1933	Novak and Henrici	Pleomorphic organism between staphylococcus and actinomycete.	Old broth cultures.
1933	Rettger and Gillespie	<u>B. megatherium</u>	Serial transfers in lithium chloride broth at 22 C.
1934	Koser and Dienst	<u>Shigella paradysenteriae</u> Sonne.	Ageing casein-digest cults. Old agar slopes.
1934	Raney and Kopeloff	<u>Lactobacillus acidophilus</u>	Old broth cultures.
1934	Roe	<u>Clostridium welchii</u>	Ageing colonies and broth cultures.
1934	Roos, Reichel and Clark	Haemolytic streptococcus	Brain broth
1936 a, b	Chinn	<u>Shigella paradysenteriae</u> Sonne  <u>Staphylococcus aureus</u>	

E X P E R I M E N T A L

Origin of the G-type.

The present thesis reports the isolation of an undoubted G-type variant of B. paratyphosus B (Tidy) which appeared spontaneously and coincident with certain dissociative reactions occurring in the laboratory strain of this organism. In passing, it must be pointed out that while this variant has been found to conform to Hadley's description in almost every particular as regards colony-characters, morphology, biochemical activity, etc., the term "G-type" in this paper is used mainly for convenience in reference, and to indicate essential identity with Hadley's variant. On the other hand it must not be assumed that all its original implications (especially as to bacterial life cycles) can be held to apply in the present use. This and other questions receive full treatment in another section.

Unusual dwarf colonies were first observed over four years ago on a MacConkey plate which had been inoculated 48 hours previously from a broth culture of B. paratyphosus B. Lying alongside normal S types, they were fairly numerous, and the majority of a size just visible to the naked eye. Their appearance under the plate microscope is shown in

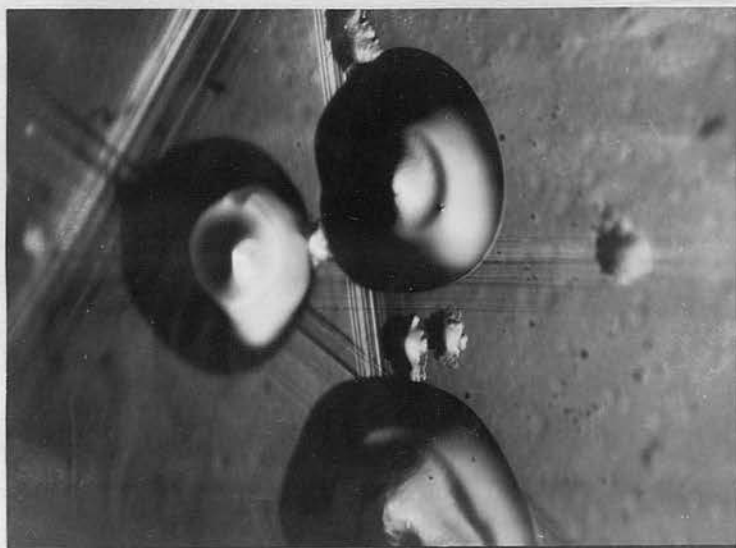


Fig. 4. Normal S colonies of  
B. paratyphosus B (Tidy)  
with transitional dwarf  
variants.



Fig. 5. Normal S type colonies  
of B. paratyphosus B (Tidy)  
with transitional dwarf  
variants.

Figs. 4 and 5. From facts which emerged later and from the subsequent behaviour of the variant, this early form may be referred to as the T (transitional) colony intermediate between the S, R or SR forms and the G form. Subculture to broth was not attempted for several days, during which time the plate was left at room temperature. Cover-glass impressions of this type of colony, fixed in alcohol and stained in dilute Giemsa after the method described by Ledingham (1933) showed large numbers of degenerate bacilli, staining unevenly and having in many cases an irregular granular structure. Apart from these bacillary forms, the mass of the colony was made up of fine granules, while here and there were seen long filamentous or tubular structures radiating from the centre. (Fig. 6).

The first attempts to subculture were unsuccessful. Twenty colonies were picked off with a fine platinum needle --- under the Leitz plate microscope with arm rests --- and inoculated individually to 5 c.c. amounts of nutrient broth, but no evidence of growth could be detected after 10 days of incubation at 37° C. Similarly, no growth was obtained on transfer of the colonies directly to solid medium.

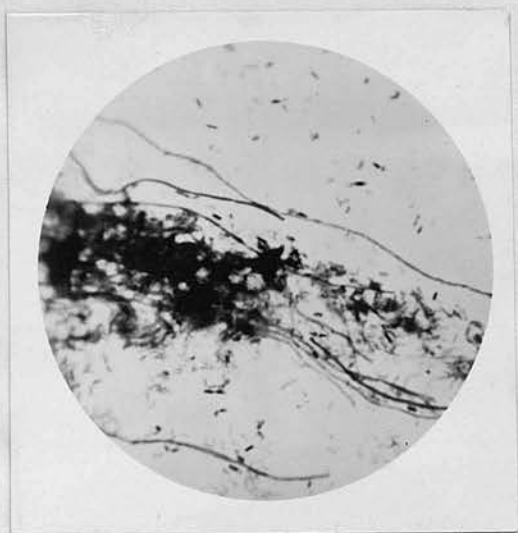


Fig. 6. Impression film  
from transitional  
colony. x 800.

Meanwhile the original broth culture of B. paratyphosus B was re-plated. The same transitional variant appeared after 48 hours, and colonies were at once touched off to broth in the same manner as before. Growth occurred in every case after 24 hours incubation, and thereafter subculture to broth was carried out daily. Examination of broth cultures at this stage showed exclusively Gram-negative bacillary forms, fragmented and granular and showing the phenomenon of lateral beading (Fig. 7).

Re-plating of such a broth culture to MacConkey's medium or to nutrient agar gave rise to a mixture of SR, R and transitional dwarf colonies. The former were not studied in detail, but various abnormal types were noted, the appearance of which suggested that the dwarf forms may originate from a disintegrative change commencing during dissociation in the periphery of colonies of SR or R type.

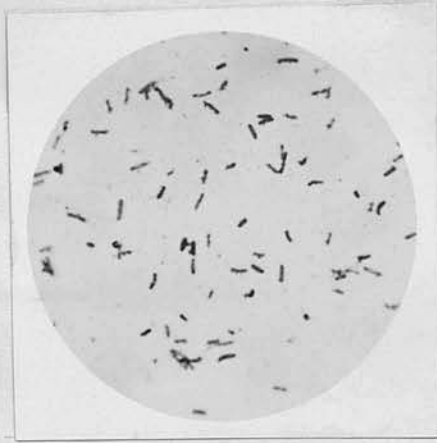


Fig. 7. Morphology of  
organisms from  
transitional colony. x 650.

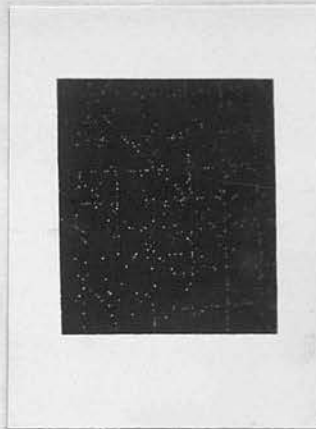


Fig. 8. Stable culture of  
G type. x 1.

Filtration experiments.

Parallel filtrations were commenced at this stage, a sample of the broth culture being filtered through a British Berkefeld candle every 48 hours. The pressure within the filtration flask was on no occasion reduced to less than  $\frac{1}{2}$ -atmospheric, and in all filtrations after the third the candle was simultaneously tested by the addition of a suspension of B. prodigiosus to the culture. Immediately after filtration, 4 or 5 drops of filtrate were seeded to a recently-poured nutrient agar plate and spread with a sterile glass rod; the remainder of the filtrate was at once transferred to sealed tubes for storage at room temperature. It should be noted that from this point onward nutrient agar was used as the routine solid medium for cultivation in these filtration experiments. Where a plate appeared sterile after 24 hours, the surface was carefully washed with 2-3 c.c. sterile broth and the washings re-plated in the same manner. Certain of these filtration results are shown in Table II.

T A B L E II

Berkefeld filtrations of broth cultures of the transitional dwarf variant.

Filtration No.	Result of filtrate plating	Subsequent behaviour of filtrate at room-temperature
I	Immediate recovery (24-hour) of typical <u>B. paratyphosus B</u> (S type) in large numbers.	Commencing cloudiness within 24 hours.
II	" " "	" " "
III	As in I and II, with in addition a few dwarf colonies	" " "
IV	" " "	Cloudiness commencing after several days.
V	First plate apparently sterile, washings transferred to second plate yielded typical G-type colonies.	Slow appearance of granular deposit, after many days.
VI	First plate showed surface growth of film, and cols. of G-type.	" " "
VII	" " "	" " "
VIII	Surface film on first plate. Washings to second plate gave film and typical G colonies.	Filtrate clear after 8 weeks.

T A B L E II continued.....

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Filtration No.	Result of filtrate plating	Subsequent behaviour of filtrate at room-temperature.
IX	First and second plates apparently sterile. Washing to third plate gave typical G-colonies.	Filtrate clear after 8 weeks.
X	G colonies recovered on third plate.	" " "

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In the course of work with Berkefeld candles it became clear that no great reliance could be placed on results in any one isolated filtration experiment. It now seems clear that really critical data can only be obtained by the use of tested collodion membrane-filters having a known range of pore-size. These methods were not applied in the present case, which indeed has not been studied from the point of view of filterability of the G elements primarily. Nevertheless enough work was done to indicate clearly that the G-type organism will consistently pass through Berkefeld candles, as stated in Hadley's experiments.

#### Origin of the typical G-type colony.

Although immediate reversion is a possibility, the results of filtrations I - IV in Table II are most readily explained on the basis of leakage of a few paratyphoid bacilli through the candles. In filtrations V to X on the other hand the organism recovered conformed to all the main criteria of the G-type described by Hadley, as will be seen later. It appears from these results that the transitional dwarf colony contained a mixture of paratyphoid bacilli and G-elements, and that these filtrations were effective in separating the two forms.

The minute colonies which appeared for the first time in pure culture after filtration V are shown in Fig. 8. Films and impressions from these colonies showed them to contain coccal forms alone, and broth cultures from such colonies also contained this element exclusively, in both staphylococcal-like groups and clusters and short chains or streptoformations (Fig. 9). These coccal elements were mainly Gram-negative, although they showed a slight degree of resistance to decolorisation, in contrast to the granular bacillary or the normal paratyphosus forms; in certain films individual cocci were seen to give a weak Gram-positive reaction. From the comparison of these facts with the descriptions given by Hadley, there can be little doubt that this organism represents the G-variant of B. paratyphosus B.

#### Morphology of transitional and G-type colonies.

While the transitional colony originally observed was rough in contour and irregular in outline (Figs 4, 5), the typical G-colony which appeared for the first time in filtrations V to X was smooth, circular and almost transparent, slow in growth and varying in size from those just visible to colonies 1.0 mm. in diameter (Fig. 8). While the average size was approximately 0.4 mm., certain

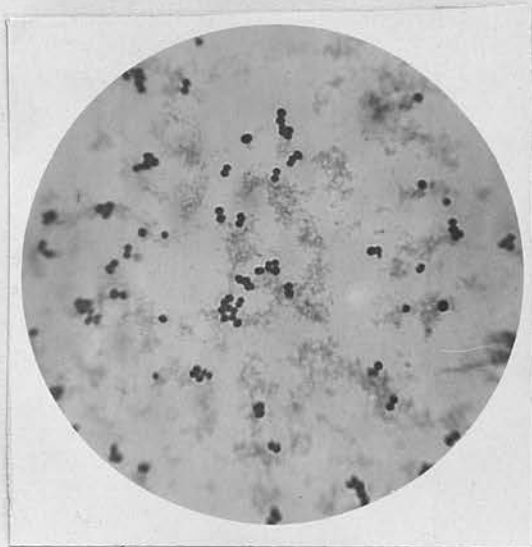


Fig. 9. Morphology of  
the G type coccus.  
x 1000.



Fig. 10. Morphological changes  
in ageing G-type  
colonies.

colonies allowed to grow for 10-14 days at 37 C. reached a maximum diameter of 2 mm. and underwent certain morphological changes (Fig. 10). An additional feature occasionally found in plates spread from broth was an almost invisible and finely granular surface film of growth, apparently made up of multitudes of microscopic colonies.

Conditions of growth and biochemical reactions of the G-type cultures.

Pure cultures in nutrient broth showed only a faint turbidity even after many days of incubation. This was frequently accompanied by a slight flocculent or granular deposit. In phosphate broth a marked turbidity resulted after 24 hours. Although the organism can be described as an aerobe and facultative anaerobe, no growth occurred in the most complete degree of anaerobiosis obtainable in the McIntosh and Fildes apparatus; growth was immediately resumed when these anaerobic preparations were restored to aerobic conditions. Growth appeared in gelatin stab cultures, after many days at 15° C., in the form of white spherical colonies (c. 1.0 mm. in diameter) along the track of inoculation; these were much more numerous at the surface of the gelatin, and no liquefaction occurred. Colonies appeared readily on nutrient agar plates, rather less readily

on MacConkey's medium, where they gave rise to active lactose-fermentation. Rapid green-production with alpha-haemolysis appeared in stroke inoculations on blood-agar plates. Definite turbidity resulted in Robertson's bullock-heart medium after several days of incubation. Growth in peptone-water was either absent or slight, and in the latter case a flocculent deposit appeared after some days. No production of indole was detected. No blackening of lead acetate agar occurred. In fluid medium glucose, lactose and saccharose were actively fermented with the production of acid only, while no change occurred in dulcitate, mannite or xylose. These reactions remained perfectly constant on every occasion on which they were tested, and obviously represent a wide divergence from the reactions characteristic of the normal type of B. paratyphosus B.

#### Thermo-resistance of the G-type.

The G-type was evidently more highly resistant to heat than the standard paratyphoid bacillus, and living organisms were regularly present after long exposures of several hours in the water-bath at 55<sup>o</sup> C. The results obtained after exposure to higher temperatures were markedly irregular and suggested some loss of heat-resistance of the organism after

long sojourns in broth.

Bacteriophage and the G-type.

A polyvalent bacteriophage active against many members of the Gram-negative intestinal group, and strongly active against various strains of B. paratyphosus B, was quite inactive when tested on the G-variant. This finding proved perfectly constant on repeated tests made at different times throughout the whole study, and is in keeping with the phage-insensitivity reported by Hadley for various G-types, and by Hoffstadt and Almaden (1934) for the G-type of Staphylococcus aureus.

Virulence and toxicity.

No experiments were carried out expressly to test these characters, but in the preparation of agglutinating antisera rabbits received large and repeated intravenous doses of live cultures without apparent harm. On autopsy, such animals showed no pathological lesions in any organ examined, and sterile cultures were obtained from liver, spleen, kidney and blood.

Serological relationship between the G-type and  
B. paratyphosus B.

An anti-G-type agglutinating serum was obtained by the repeated intravenous administration of live broth cultures in rabbits. To prepare an emulsion of suitable density for the agglutination reaction, 60 c.c. of broth culture of the G-variant was centrifuged at 2,500 r.p.m. and the deposit taken up in 3 c.c. saline; this gave a sufficiently dense yet stable suspension. Reactions were carried out at 37°C. for several hours, although in later work much clearer readings were obtained by leaving the tests at 55°C. for an hour and reading immediately. The reactions between the G- and S-phases of B. paratyphosus B are shown in Table III.

T A B L E III

Agglutination reactions between B. paratyphosus B antisera (anti-G, anti-S), and G and S antigens.

SERUM	Anti- :gen (0.4 c.c.)	Serum dilutions.....0.4 c.c.						CONTROL
		1:30	1:60	1:120	1:240	1:480	1:960	
Anti-S	G *	-	+	+++	++	-	-	-
	S	+++	+++	+++	+++	+++	+++	-
Anti-G	G	++	++	++	++	++	++	-
	S	-	-	-	-	-	-	-

\* prezonal reaction.

Thus the G serum had no effect on the S antigen but agglutinated well its own antigen, while there was some hint of the presence of co-existent G antigen in the original S antigen used to prepare the anti-S serum. This agglutination of the G organism by an anti-S serum never proceeded to end-titre, and in several

later tests no cross-agglutination of any kind could be detected.

Subsequent behaviour of the G-type.

After primary isolation as described above, the G variant was subcultured in ordinary broth at intervals of 1-4 days. In the early stages about 0.5 c.c. of culture was taken to inoculate each fresh broth tube (containing about 7-8 c.c.), but in later transfers a single loopful only was used. At each transfer the corresponding broth culture was plated on nutrient agar and on MacConkey's medium, and the resulting colonies examined at intervals for signs of variation. In the course of the investigation the original G strain underwent 260 such serial transfers and platings. During this period there were no signs of mass-reversion to the original parent-type, although discontinuous variants of considerable interest appeared from time to time. Attempts were made to accelerate reversion by repeated selection of G-colonies of larger size or increased opacity, but without result. With the same end in view the G organism was cultivated in 200 c.c. volumes of ordinary broth and of broth containing 10 per cent. specific immune serum, but repeated platings at short

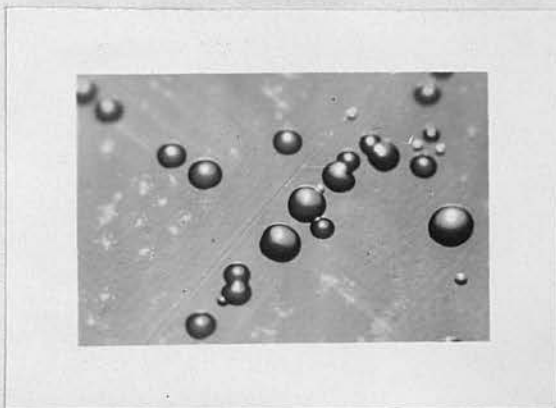


Fig. 11. Size variation in  
G colonies.

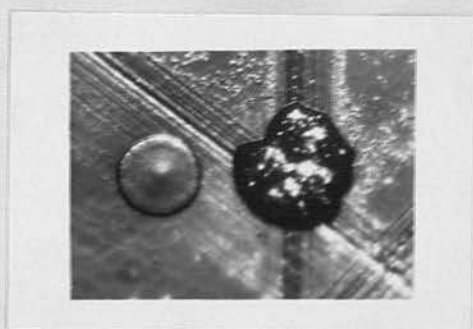


Fig. 12. Morphological changes in  
G type colonies in  
process of ageing.



Fig. 12a. As for Fig. 12.

intervals showed no indication of departure from the typical characters described above. Before describing the mode of origin and nature of the discontinuous G-derived variants already referred to, mention may be made of two temporary types of variation in the G strain which were observed from time to time. The first manifested itself by a wide variation in the size of individual colonies (Fig 11), and the second by the appearance of colony roughness and the development of ~~large numbers of~~ papillae (Figs 12 and 12a).

#### Isolation of discontinuous variants.

Strain G 41. On an agar plate inoculated from the 41st broth transfer of the original G strain, several G colonies were seen to be growing much more rapidly than the majority of the colonies present (Fig. 13). On investigation, the growing edge of these colonies was found to be composed of gram-negative bacilli, while the organisms in the centre were cocci of the G type. This colony type was isolated in pure culture and maintained as a separate strain. It appeared to differ from the G-type mainly in its more rapid growth-rate, and in the appearance for the first time of bacillary morphology. The fermentation reactions remained unchanged from those of the G-type. Although it remained serologically distinct from

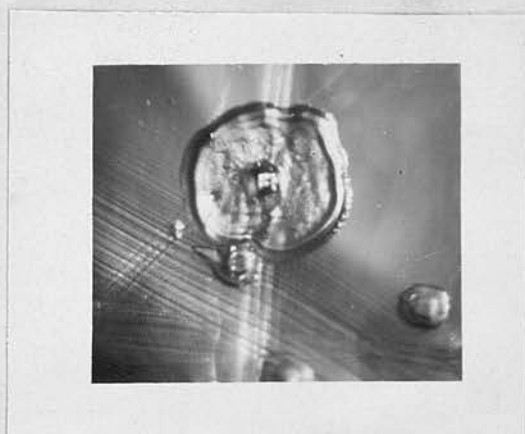


Fig. 13. Increase in size  
of individual G  
colony to give strain  
G 41.

A

B

C

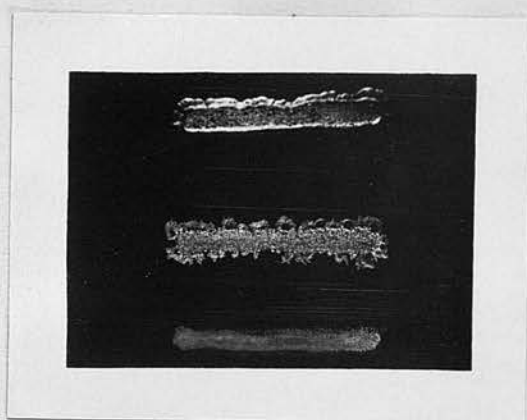


Fig. 14. Comparison between stroke inoculations of (A) *B. paratyphosus* B (white, bacillary, extending beyond inoculation area; (B) strain G 41 (coccal in area of inoculation, slight green production, extending feathery edge of Gram-negative bacilli); and (C) the G strain (coccal, limited to area of inoculation, green production marked)..... on boiled-blood-agar. Slightly reduced. Appearance on 1st day after inoculation.

A

B

C

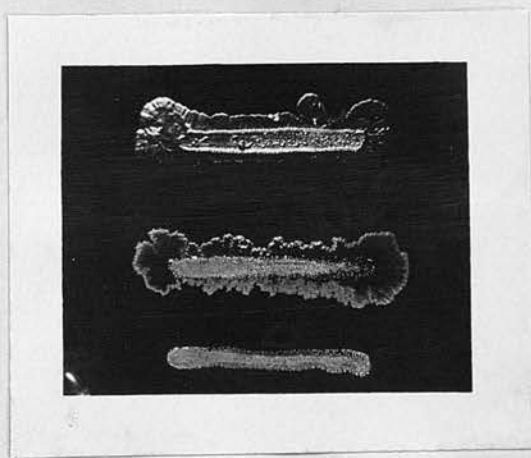


Fig. 15. As for Fig. 14; appearance one day later.

B. paratyphosus B and insensitive to bacteriophage, as did all the bacillary variants recovered, strain G 41 appeared nevertheless to exhibit certain characters intermediate between those of the G-type and the paratyphoid bacillus. Thus while single stroke inoculations of the paratyphoid bacillus on boiled blood agar gave a grey or white line of growth extending later as a raised or rolled edge, and similar inoculations of the G organism resulted in growth and green-production limited to the inoculation area, such an inoculation with strain G 41 gave at first a localised area of growth (coccal), with slight green-production, followed later by the appearance of a grey and rapidly-growing bacillary edge (Figs 14, 15).

All attempts to isolate the bacillary form in stable culture failed. In broth culture it rapidly reverted to the coccal form, and at all times such broth cultures when plated yielded a mixture of typical G colonies and of coccal colonies in which bacilli appeared as the colony increased in size. The tendency of strain G 41 to revert to the G-type increased with serial transfer in broth, and after repeated transfer the strain ceased to yield bacillary forms and became indistinguishable from the original G strain from which it was derived. Intermediate colony types in this process of reversion are shown in Figs. 16-22.

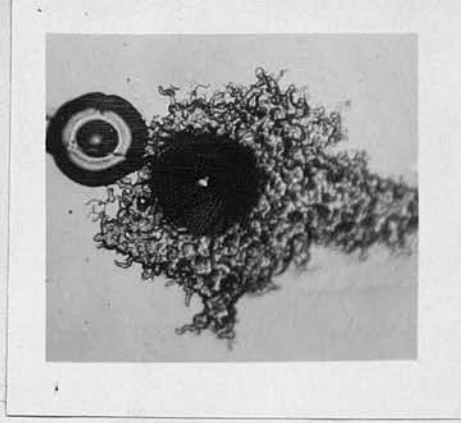


Fig. 16

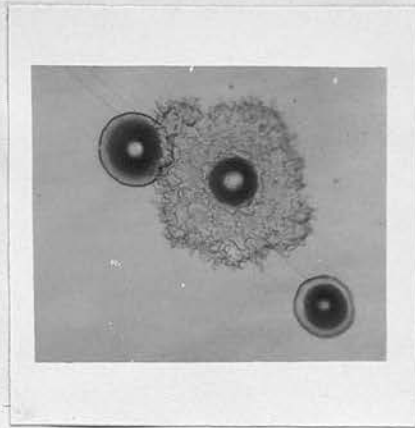


Fig. 17

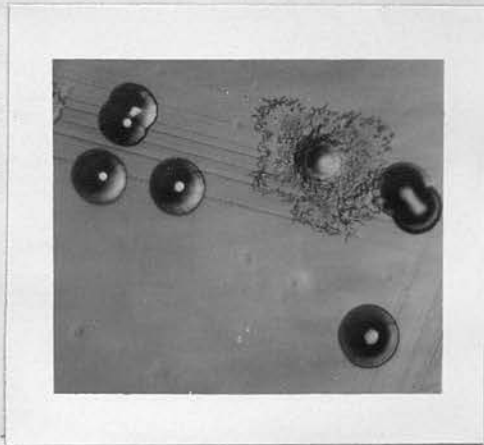


Fig. 18

Figs. 16, 17, 18. Types of variation in Strain G 41.

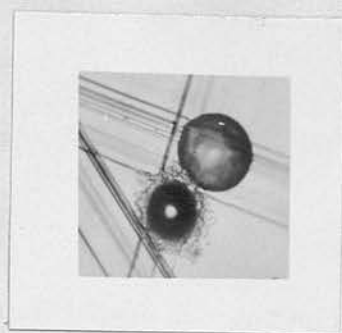


Fig. 19 x 15

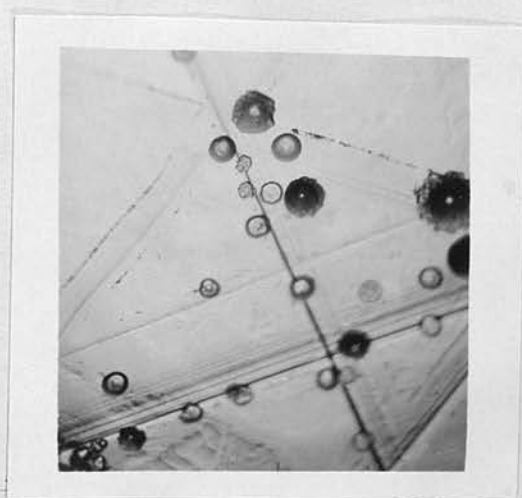


Fig. 20. x 15

Figs. 19, 20. Types of variation in Strain G 41.

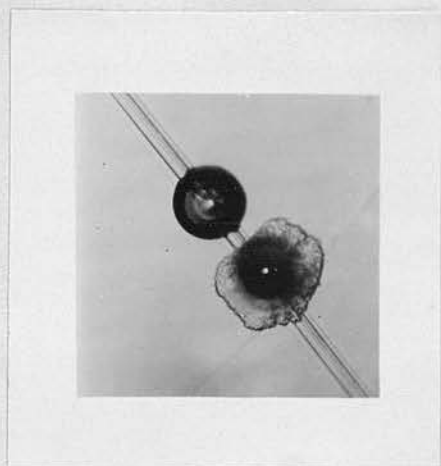


Fig. 21

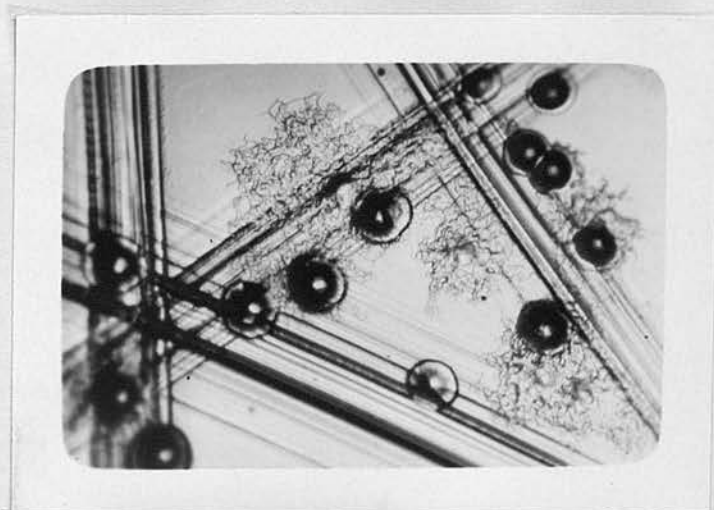


Fig. 22

x 25

Figs. 21, 22. Types of variation in Strain G 41.

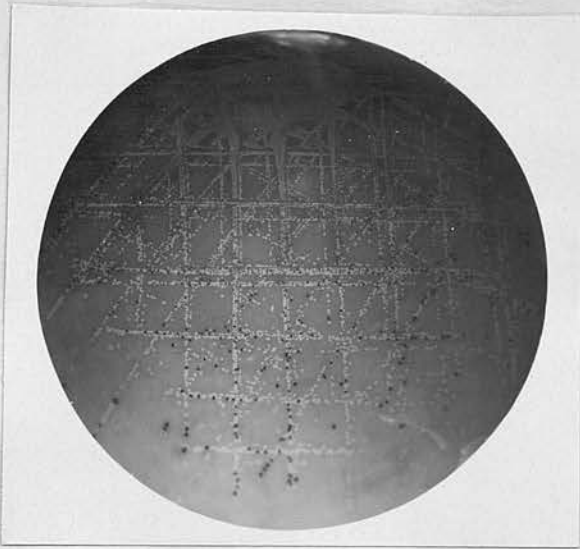


Fig. 23. Emergence of  
non-lactose fermenting  
variant of G 41. Slightly  
reduced.

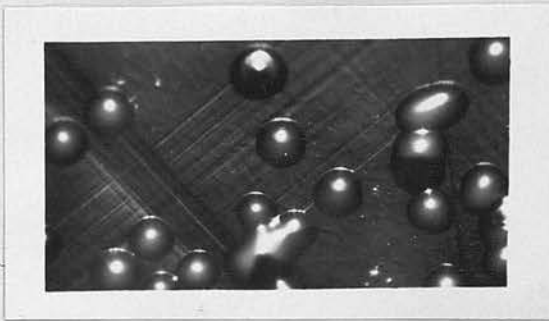


Fig. 24. As above. x 30.

Strain G 41/34.

When the 34th broth transfer of strain G 41 was plated on MacConkey's medium, certain small G-type colonies were seen which appeared not to ferment lactose (Figs. 23, 24). This variant was isolated as a separate strain (G 41/34). It resembled the original G strain in every particular with the exception that no acid-production could be detected in glucose or lactose even after many days of incubation. Active acid-production occurred however in saccharose within 24 hours.

Strain G 41/34/24.

On plating the 24th broth transfer of strain G 41/34 to agar, it was found that the plate rapidly became covered with a film of growth due to the emergence of a motile bacillary form (Figs <sup>25, 25a</sup> 24, 25). Spreading was completely inhibited on MacConkey's medium. This variant was maintained as a new individual sub-strain (G 41/34/24). Morphologically it was an actively motile Gram-negative bacillus of coliform type, and its chief biochemical characters, contrasted with those of the original G strain, are shown in Table IV.

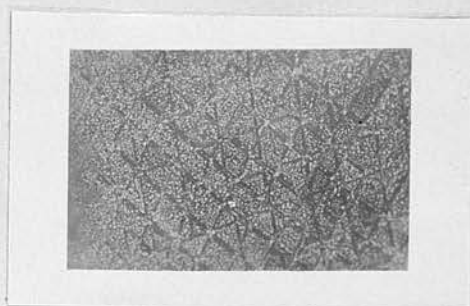


Fig. 25. Swarming growth  
of strain G 41/34/24.  
x 1.

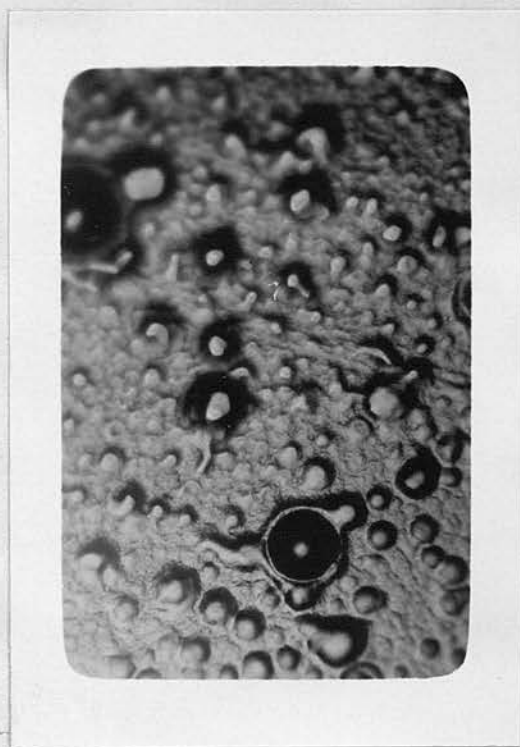


Fig. 25a. As for Fig. 25.  
x 40.

T A B L E I V

	Morphology	Motility	Glucose	Lactose	Saccharose	Dulcitate	Mannite	Xylose	H <sub>2</sub> S-productn.	Indole-productn.
<u>Strain G.</u>										
coccus	-	✓	✓	✓	-	-	-	-	-	-
<u>Strain G 41/34/24.</u>										
bacillus	+	✓	✓	✓	-	-	✓	✓	-	-

✓ = acid-production.

Strain G 41/34/24/69.

The spreading character of strain G 41/34/24 gradually became less marked in the course of serial cultivation in broth. After about 50 transfers at intervals of a few days the plates began to show discrete bacillary colonies with no tendency to spread even after prolonged incubation. One such colony was picked off from the agar plate inoculated from the 69th broth transfer, and this was maintained as sub-strain G 41/34/24/69. Apart from complete loss of motility this organism was identical in all its characters with the Gram-negative bacillus of strain G 41/34/24 (see Fig. 26).

Strain G 74F.

The agar plate inoculated from the 74th broth transfer in the original G strain was found to develop a rapidly spreading film of growth identical with that described above in the case of strain G 41/34/24. The spreading was again due to a Gram-negative bacillus which grew in the form of a film and in addition gave rise to localised "swarm-colonies" of varying size (Figs. 27, 28). This effect was completely inhibited on MacConkey's medium, where inoculation with this strain gave rise to G-type colonies alone (Fig. 29).



Fig. 26. Organism from  
strain G 41/34/24/69.  
x 1000.

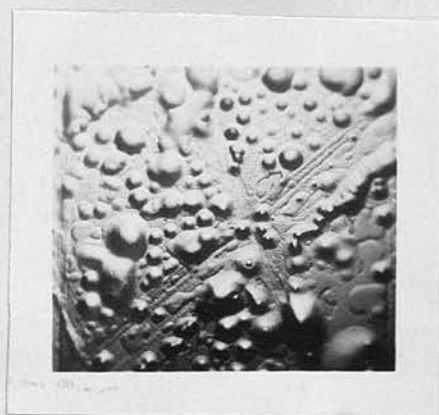


Fig. 27. Swarming in strain  
G 74F.

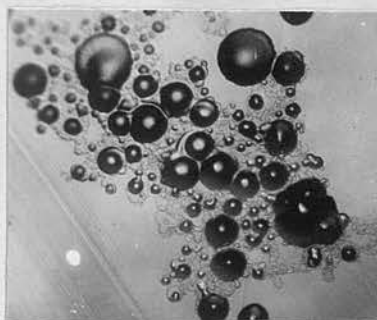


Fig. 28. Swarming in strain  
G 74F.



Fig. 29. Colonies from  
strain G 74F grown  
on MacConkey's medium.

Strain G 74/48.

As in the case of strain G 41/34/24 already described, the swarming effect characteristic of strain G 74F gradually lessened in successive broth transfers, a change probably due again to a loss of motility on the part of increasing numbers of bacilli. Individual colonies on agar became more and more discrete and free from spreading, and one such colony from the 48th transfer of strain G 74F was picked off to broth as strain G 74/48. This organism was found to be practically identical with the bacillus of strain G 41/34/24/69 as regards morphology, cultural characters, fermentation reactions and serology (vide infra; and Figs 30-34).

Strain G 74/48/15.

On primary isolation strain G 74/48 grew with the production of moderate and uniform turbidity in broth, and gave rise to exclusively smooth colonies on nutrient agar. Dissociative changes of the S----->R type became evident after a few broth transfers, (see also Figs. 30-34), and the R variant was isolated as a separate strain, G 74/48/15. In broth it produced a flocculent type of growth near the surface, and was not readily emulsifiable in saline. (Figs. 35, 36).

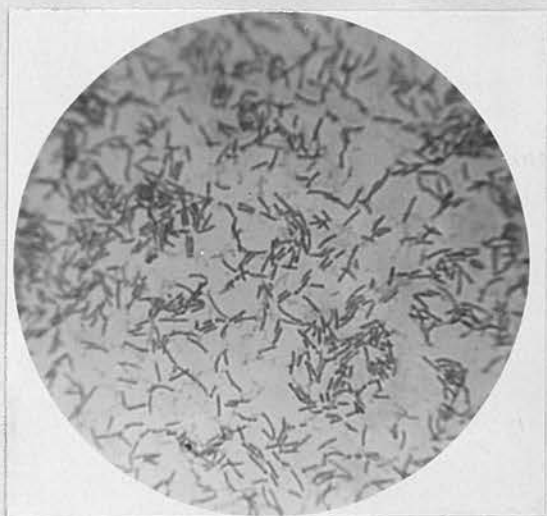


Fig. 30. The organism of  
strain G 74/48.  
x 1000.

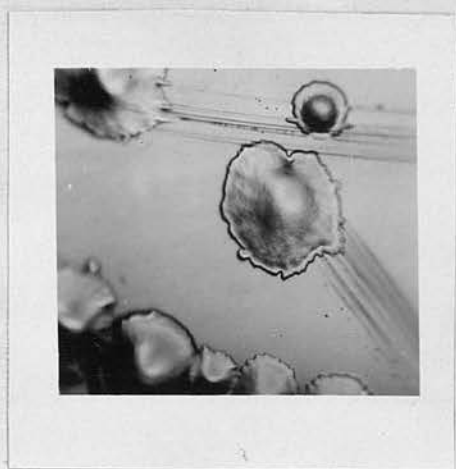


Fig. 31.

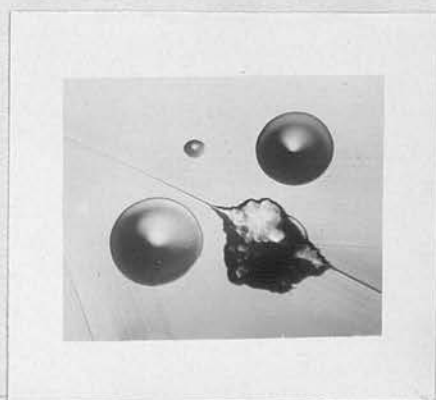


Fig. 32.

Figs. 31, 32. Dissociative types in  
Strain G 74/48.



Fig. 33.

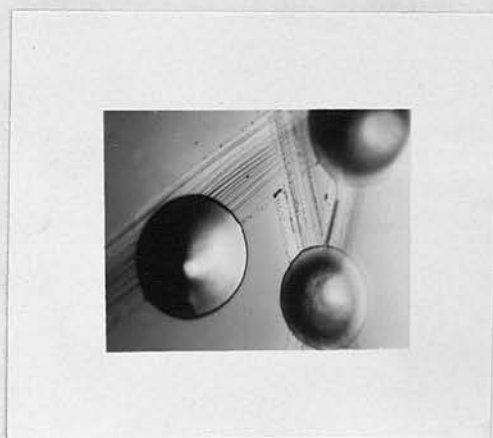


Fig. 34.

Figs. 33, 34. Dissociative types in  
strain G 74/48.

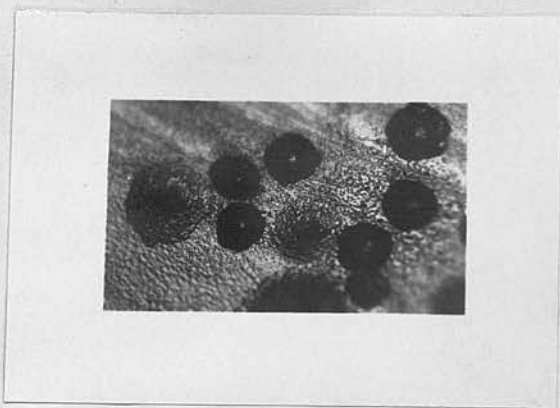


Fig. 35.

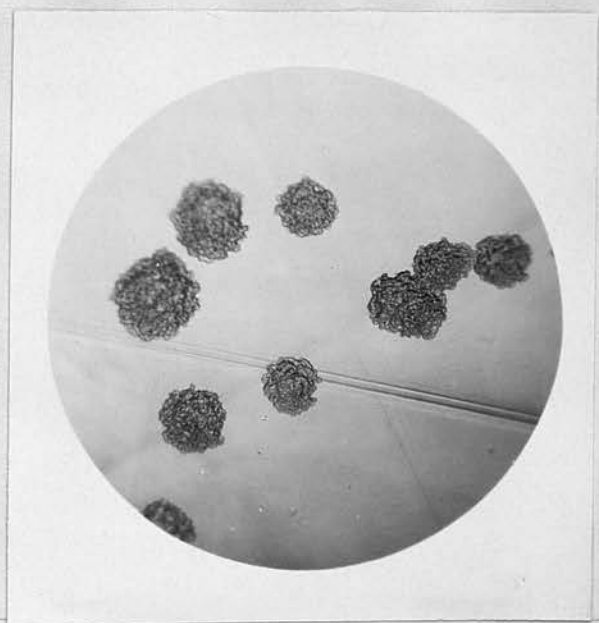


Fig. 36.

Figs. 35, 36. The bacillary variant  
G 74/48/15.

Strain G 74/30.

On a MacConkey plate inoculated from the 30th broth transfer of strain G 74F, it was observed that large numbers of the resultant G-type colonies had lost their capacity for lactose-fermentation. The non-lactose-fermenting type was isolated in pure culture and proved to be stable in respect of this new characteristic (strain G 74/30).

Strain G 74/30/73.

The 73rd serial broth transfer of strain G 74/30 was found to have developed unusual increase in turbidity after one month at 37<sup>o</sup> C. Plating this culture on agar resulted in a mixture of small non-lactose-fermenting colonies of bacillary type. It proved to be a non-motile Gram-negative bacillus growing in filaments. Its main biochemical reactions, together with those of the strains already described and those which follow, are given in Table V.

Strain G/158.

On plating the 158th serial broth transfer of the original G strain several colonies were observed to be growing much faster than the majority, although presenting no other departure from the G-type as



Morphology Motility Glucose Lactose Saccharose Dulcitate Mannite Xylose H<sub>2</sub>S-productn. Indole-productn.

Strain G 41/34/24.

bacillus + ⊥ ⊥ ⊥ - ⊥ ⊥ - -

Strain G 41/34/24/69.

bacillus - ⊥ ⊥ ⊥ - ⊥ ⊥ - -

Strain G 74/30.

coccus ⊥ - ⊥ - - -

continued over leaf.....

Morphology Motility Glucose Lactose Saccharose Dulcitate Mannite Xylose H<sub>2</sub>S-  
productn. Indole-  
productn. productn.

Strain G 74/30/73.

bacillus - ⊥ - ⊥ - ⊥ - -

Strain G 74F.

bacillus + ⊥ ⊥ ⊥ ⊥ ⊥ ⊥ - -

Strains G 74/48 and 74/48/15.

bacillus - ⊥ ⊥ ⊥ ⊥ ⊥ ⊥ - -

continued overleaf.....

Morphology	Motility	Glucose	Lactose	Saccharose	Dulcitate	Mannite	Xylose	H <sub>2</sub> S- productn.	Indole- productn.
<u>Strain G/158.</u>									
cocco- bacillus	-	⊥	⊥	⊥	-	-	-	-	-
<u>Strain Auto B.</u>									
coccus		⊥	⊥	⊥	-	-	-	-	-
<u>Strain SS-2.</u>									
bacillus	+	⊥	-	⊥	-	-	-	-	-
<u>Strain G 207L.</u>									
bacillus	-	-	-	-	-	-	-	-	-

⊥ = acid-production.

regards colony appearance. Several colonies representative of this variant were picked off to agar slopes, where they grew much more readily than did the original G-type under such conditions. Films showed the variant organism to be a short Gram-negative coliform bacillus or cocco-bacillus, and further investigation showed its fermentative reactions to be unchanged from those of the original G strain. It was thenceforth maintained in serial transfer in broth as a separate strain G/158. From the beginning it was evidently rather unstable as regards its new characters of rapid growth and assumption of bacillary form, and showed a constant tendency to revert to the original G-type (Figs 37, 38).

Fig. 39 shows the appearance on microscopic examination, a mixture of Gram-negative filamentous rods, cocci and cocco-bacilli.

#### Strain G 207L.

At the 197th serial broth transfer of the original G strain, subculture was carried out to liver broth as well as to ordinary nutrient broth. Thenceforth subculture was maintained in parallel in these media. When the tenth liver broth culture was plated on MacConkey's medium a single colony was observed to be growing more rapidly than the others and to be fermenting lactose less strongly than

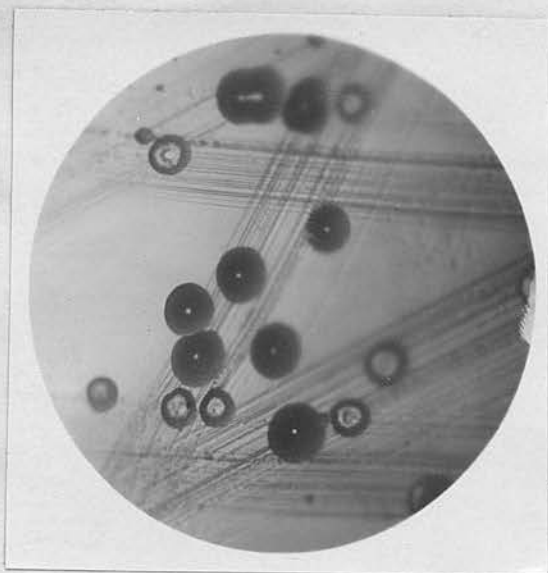


Fig. 37.

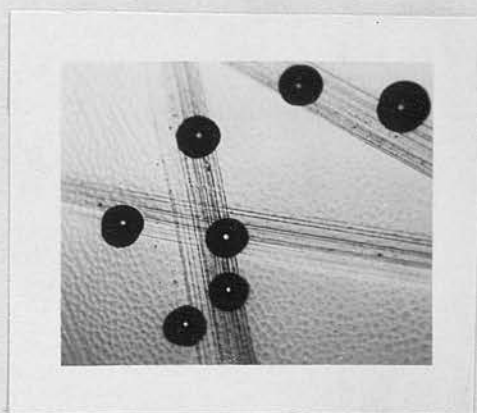


Fig. 38.

Figs. 37, 38. Strain G/158. Colonies  
showing tendency to revert  
to smaller coccal type.

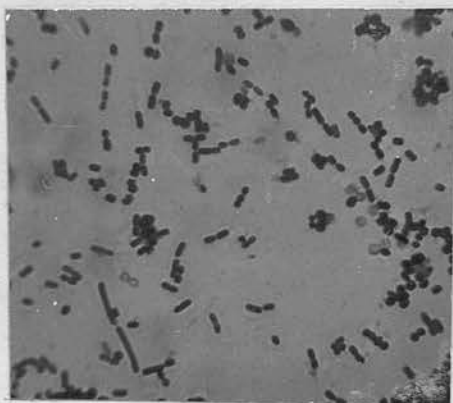


Fig. 39. Strain G/158.

x 1000.

usual. This variant was immediately picked off to broth and studied further. It was found to be a non-motile Gram-negative bacillus, and on re-plating to MacConkey's medium gave large pale colonies (Fig. 40) within 48 hours. After leaving such a plate at room temperature for a week it was found that all the colonies developed the appearance of lactose-fermentation. This plate was returned to the incubator at 37° C. for 24 hours, when it was found that the colonies had again become almost completely pale. Apart from this peculiarity in lactose fermentation, the organism was biochemically almost inactive as judged by the fermentation reactions employed in the present study. The variant was maintained in serial broth culture as strain G 207L. Its subsequent behaviour remained unchanged apart from variability in acid-production on MacConkey's medium at 37° C; while the colonies under these conditions were usually pale, as described, occasionally there were indications of lactose-fermentation at 37° C. as well as at room-temperature.

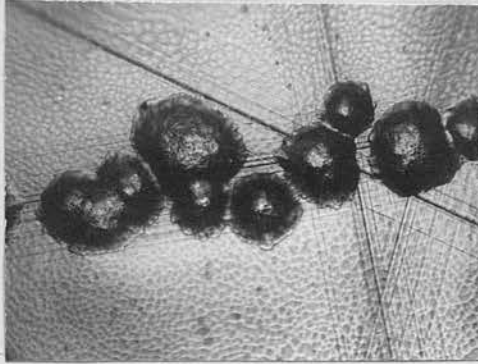


Fig. 40. Strain G 207L.

Strain SS2.

This organism did not appear spontaneously but was recovered in the course of an experiment to determine whether reversion in the G strain could be induced by growing the G coccus in the presence of killed paratyphoid bacilli. Although no lytic agent or bacteriophage active against B. paratyphosus B could be demonstrated in G-type cultures, it was felt that the non-occurrence of reversion in the G-type might be due to the presence in the G-cultures of an agent inhibitory to the emergence of the paratyphoid bacillus, i.e., to complete reversion. An experiment was therefore performed on the lines suggested by Kendall and Walker (1933) for the recovery of non-filterable bacteria from the filterable state in active bacteriophage filtrates. In one of these experiments Kendall and Walker employed the methods of dilution and of addition of killed homologous organisms in order to curb phage-action; under these circumstances the viable and filterable elements present in the bacteriophage filtrates easily reverted to the fully-developed non-filterable state.

In the case under discussion 1 c.c. of a 24-hour culture of the G strain was added to 9 c.c. of broth; decimal dilutions were then made by transferring 1 c.c. from the preceding tube in all of a series of 9 tubes each containing 9 c.c. broth. To every tube was then

added 1 c.c. of a culture of B. paratyphosus B (Tidy) which had been grown on agar for 24 hours, washed off with sterile broth, and autoclaved for 20 minutes at 120° C. The tubes were then incubated at 37° C. and platings carried out every 24 hours. For the first three days, plating gave typical G colonies from every tube. On the fourth day an obvious increase in turbidity manifested itself in seven of the ten tubes. Plating from these tubes gave either a pure culture of a motile Gram-negative non-lactose-fermenting bacillus (Figs. 41, 42) or a mixture of this with G colonies. The new variant was isolated as strain SS2. Its chief biochemical features, contrasted with those of the G-type and other variants, are shown in Table V.

In this experiment a similar series of tubes was incubated to control the effect of dilution of inoculum on the growth of the G-strain; that is, no autoclaved paratyphosus B culture was added. Throughout the experiment this control series gave cultures of the G-type alone. Unfortunately, at this stage no simultaneous series was run to control the effect of autoclaving on the paratyphosus cultures used. It is thus impossible to be definite regarding the precise mode of origin of the SS2 variant, and the experiment was not repeated with success. The subject will be referred to later in the discussion.

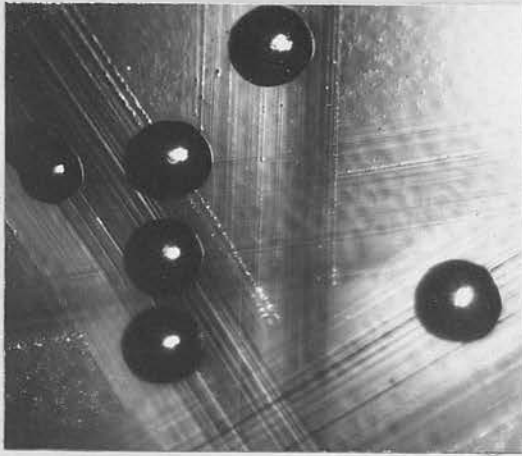


Fig. 41.

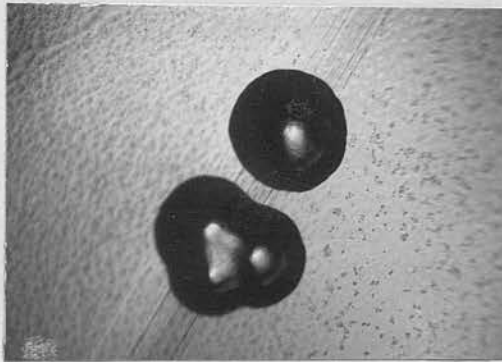


Fig. 42.

Figs. 41, 42. Strain SS-2.

Strain Auto B.

In attempts to repeat the above result and determine the exact source of strain SS2, controls were repeatedly examined to test the effect of autoclaving (at 15 lbs. for 20 minutes) on broth suspensions of agar-grown B. paratyphosus B (Tidy). Each control consisted of ten tubes of broth in 9 c.c. amounts to each of which had been added 1 c.c. of the autoclaved culture. These were incubated at 37° C. for periods of time up to ten days and plated every 2 days. In one case slight increase of turbidity appeared in two tubes after 48 hours and in a third after 72 hours, and an organism was recovered from all of these tubes which agreed in every detail with the G-strain originally isolated more than fifteen months before. This rather surprising result was obtained in two subsequent trials, in one of which the G organism was recovered from 5 of the 10 tubes inoculated with autoclaved material. The autoclave was controlled in addition by its killing effect on B. mesentericus.

The organism recovered under these circumstances was maintained in broth culture as strain Auto B (Figs. 43-46) and proved to be serologically indistinguishable from the original G strain.

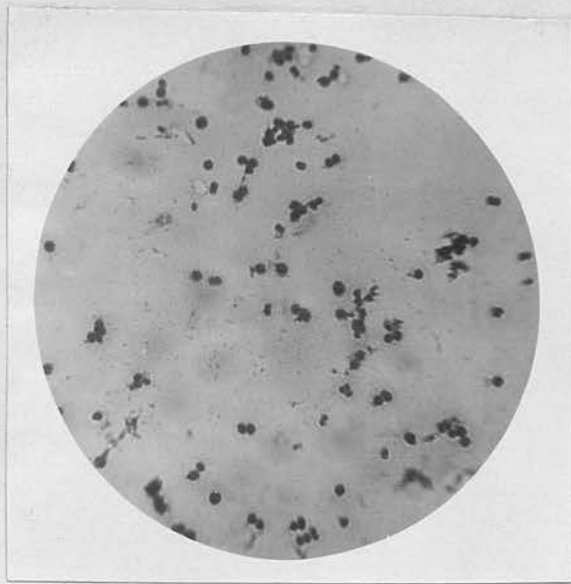


Fig. 43. Strain Auto B.  
x 1000.

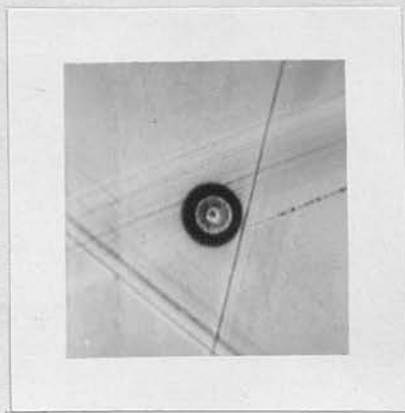


Fig. 44. Strain Auto B.  
4 days, x 30.

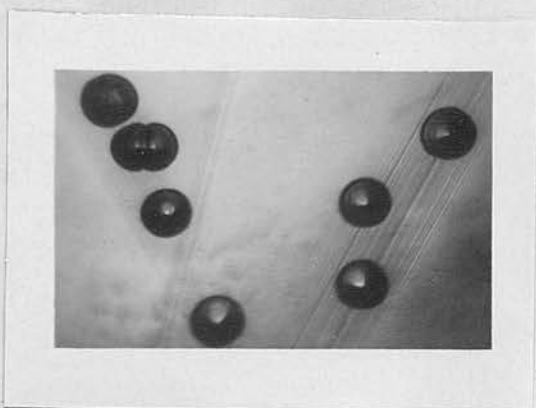


Fig. 45.

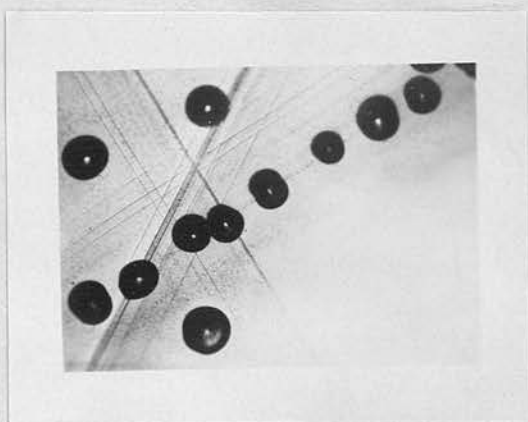


Fig. 46.

Figs. 45, 46. Colonies from  
Strain G Auto B,  
4 days, x 25.

Lactose-fermenting papillae in colonies of  
B. paratyphosus B (Tidy).

The striking phenomenon of G-variation was observed repeatedly though irregularly with the laboratory strain of B. paratyphosus B (Tidy) under three conditions: (a) on plating a broth culture of the organism after a course of serial transfers daily in broth; (b) on plating a broth culture previously heated at 55° C. for 30 minutes; (c) on plating cultures grown in broth containing 0.5 per cent. lithium chloride. In all of these experimental conditions there was also observed from time to time the production of lactose-fermenting secondary colonies when plating was carried out on MacConkey's medium. These cultures presented the usual pale appearance at the end of 24 hours growth, but shortly afterwards they were seen to be very slightly suffused with pink. This change was followed in 48 hours by the appearance on every colony of crops of deep red papillae (Figs. 47, 48). In many cases individual papillae had a striking resemblance to isolated G colonies. In spite of many attempts it was found impossible to separate the lactose-fermenting element in pure culture. Fig. 49 shows the abnormal morphological types present in such papillate colonies.



Fig. 47. Lactose-fermenting  
papillae in colonies of  
B. paratyphosus B.

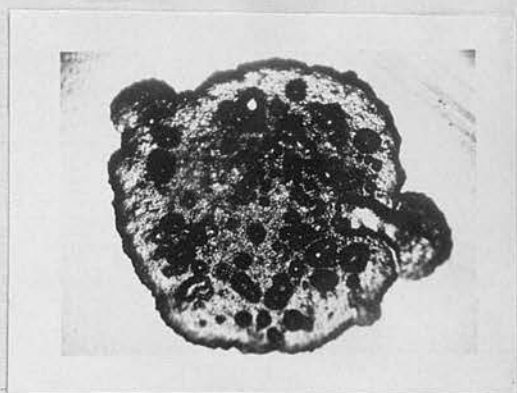


Fig. 48. As in Fig. 47.

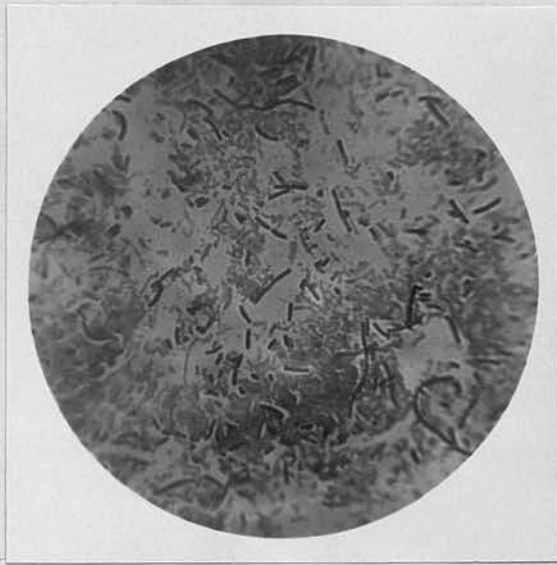


Fig. 49. Smear from papilla  
(lactose-fermenting)  
from colony of  
B. paratyphosus B(see text).

D I S C U S S I O N

Occurrence of G-type variation.

Reference has already been made in the Introduction and review of the literature to evidence corroborating the existence of so-called G-type variation in diverse bacterial species, and it has been pointed out that the recognition of this type of colony change is not entirely recent.

Jacobsen (1910) and Fromme (1911) described variants of the typhoid bacillus which grew in the form of small streptococcus-like colonies. Eisenberg (1914) drew attention to "Zwergkolonien" in Serratia marcescens and Eberthella typhi, and Baerthlein (1918) described similar forms for V. cholerae and E. typhi. Furth (1922) also obtained such Zwergkolonien from old cultures of Salmonella aertrycke and a Salmonella Schottmulleri-like organism.

While the bulk of Hadley's published work referred to G-variation in the Shiga dysentery bacillus, he and his co-workers also obtained analogous strains of G-type cultures in the following bacterial species.....B. coli (two instances); B. typhosus (seven instances); B. paratyphosus A (four instances); B. paratyphosus B (two instances); B. enteritidis (one instance); B. typhi-murium (two instances); B. typhi murium II (one instance);

B. cholerae-suis (one instance); B. acidophilus (one instance); V. cholerae (one instance); and B. diphtheriae (two instances).

Other workers have described similar appearances, and in particular Hadley (1933) discussed the relation of the bacterial variants of Kuhn to the S, R and G phases in microbial dissociation. The G forms (Kokken-Formen) of Kuhn are chiefly cocci and granular bodies, some of which are minute and filterable and whose colonies are small and sometimes microscopic. According to Kuhn they arise from the B forms (Bakterien-Formen) which comprise the ordinary rods etc. usually regarded as the normal forms of most bacterial species. Hadley regarded Kuhn's B form as probably identical with the S phase and his C form as definitely related to what have been termed the G forms.

The phenomena described in the present thesis refer to variation in B. paratyphosus B mainly, but the appearance of G-colonies was also observed in platings from B. typhosus (Cole) and B. paratyphosus A (Schottmuller), as also in a strain of B. coli (Figs. 50, 51).



Fig. 50. G-type colonies  
from a strain of B. coli.

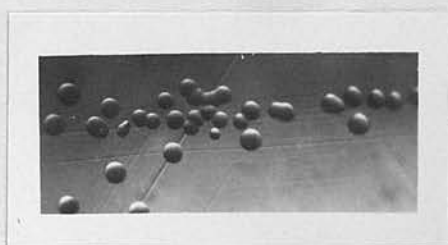


Fig. 51. As for Fig. 50.

Colony-characters of the G-type.

The colonies described as G-colonies in the present communication are closely similar to or identical in appearance with those depicted photographically by Hoffstadt and Youmans (1932) in the case of Staphylococcus aureus and by Koser and Dienst (1934) in the case of Eberthella dysenteriae Sonne. Although Hadley in his original paper (1931) provided no illustrations, his descriptions made it clear that the G-colonies, on their first appearance on plates, manifest characteristics sufficient to make certain their differentiation from at least the commonly recognised colony types such as the S, SR, R and intermediates. (See also Figs. 52-55, from photographs of G-colonies kindly gifted to the writer by Dr Hadley). Before discussing these characteristics in detail, suffice it to say that in colony appearance as in other respects the G-variants of B. paratyphosus B have conformed strictly with Hadley's original description for those of B. dysenteriae Shiga.

For purposes of description Hadley divided the G colonies on their first appearance into four groups in order of size: (1) those visible to the unaided eye; (2) those distinctly visible only with the aid of a hand lens; (3) those not distinctly visible

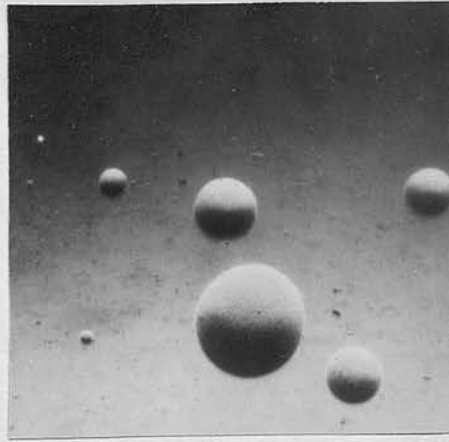


Fig. 52. G and small S colonies  
of B. dysenteriae from  
lithium chloride broth.

(from Dr Hadley)

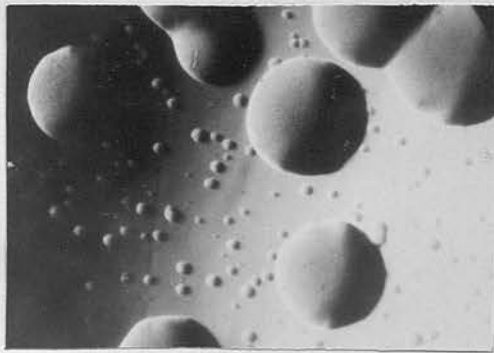


Fig. 53. As in Fig. 52.

(from Dr Hadley)



Fig. 54. G colonies produced  
by the action of lithium  
chloride on S. dysenteriae.

(from Dr Hadley)

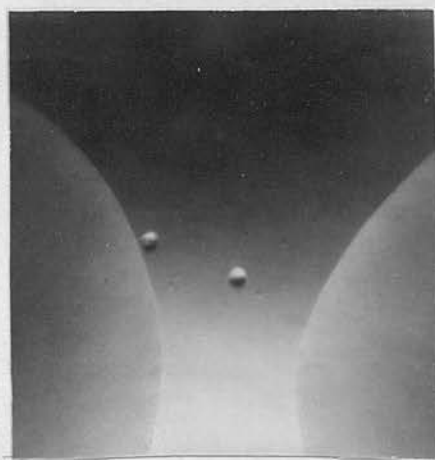


Fig. 55. G colonies produced  
by the action of lithium  
chloride on S. dysenteriae.  
Note edge of S colonies.

with a hand lens but visible with the no. 3 objective; and (4) those not visible with the no. 3 but visible with the no. 7 objective. The smallest colonies --- studied on cover-glass impression films stained by Giemsa ---had a diameter of from 0.004 to 0.006 mm. after 48 hours growth, while the largest had a diameter of about 0.2 mm. after 4 days growth. Hadley pointed out that many plates containing multitudes of the smallest colonies would have been regarded as sterile in the course of ordinary observation. Precisely the same range of colony size has been met with in the present investigation. An inherent variability in respect of colony-size would appear to be a feature of certain G strains (see above) quite independent of environmental factors such as over-crowding of colonies on the plate.

In one case Hadley described the appearance of roughness in a G strain, and this type of variation has also been observed in the present investigation (see above).

It has been noted that the G type is not easily propagable by direct agar to agar transfers but is readily grown in serial transfers in broth or on agar after a brief sojourn in broth. This also confirms a similar observation made by Hadley.

Growth of the G-type in nutrient broth.

Inoculation of nutrient broth either directly from a G colony or indirectly from a grown broth culture resulted in the appearance of a slight and homogeneous cloudiness after 24 hours. Growth under these circumstances was invariably delicate when compared with the marked turbidity of corresponding cultures of the paratyphoid bacillus itself. During the propagation of the G-form in serial broth cultures it was frequently observed that the maximum clouding was attained in 24 hours at 37° C. and was followed by the almost complete clearing of the culture within a week or ten days. Such cultures were examined, without success, for the presence of bacteriophage activity, and it seems probable that such clearing is indicative more of a spontaneous autolytic change. Standard stroke inoculations were made on agar from broth cultures of varying age kept at 37° C. Table VI shows the numbers of G colonies developing in such stroke cultures after 3 days incubation.

T A B L E VI

G-colony counts (after 3 days at 37° C.) in  
standard single stroke inoculations from  
G-type broth cultures of varying age.

---

Age of broth cult. in days	No. of G colonies
2	640
3	330
4	400
5	176
6	120
7	12
10	4

---

This autolytic process however was neither constant nor did it proceed to complete sterilisation of the culture. Aged cultures of this type were subsequently found to yield large numbers of extremely minute G colonies on prolonged incubation, suggesting that the change was more in the nature of a loss in ease of cultivation of the living elements on agar. Again, sealed and unsealed cultures of the G forms, kept at room temperature and at 37° C., were found on repeated examination to retain their

essential characteristics over many months and apparently indefinitely.

Mode of origin of the G-type.

Hadley was able to cause the generation of the G type cultures from either the S or the R forms by the following methods: (1) by serial passage through plain infusion broth of reaction pH 7.5-7.8; (2) by serial passage through lithium chloride broth; (3) by serial passage through pancreatin broth; (4) by the use of peritoneal fluid; (5) by the use of bacteriophage; (6) by ageing on agar slants, or in tubes of alkaline broth; (7) by selection of secondary G colonies in S-type mother colonies; and (8) by cultivating from the edge of old R-type colonies. His experimental methods were mainly such as induce the dissociative reaction  $S \rightarrow R$ , and he recognised three important features in the origin of the G phase. First, the emergence of the G-forms is dependent on the occurrence of coincident dissociation; secondly, the G forms make their first appearance at a certain moment, which in Hadley's experience was most likely to be between the sixth and twelfth serial tubes in a series undergoing twenty-four-hour transfers; thirdly, not infrequently the G forms appeared, remained for several passages, disappeared for many more, and



then returned again to their position among the other culture elements.

In the present study the G elements first appeared during a spontaneous phase of dissociation in the mother culture. Although subsequently this laboratory culture was repeatedly examined, the presence of G forms was very irregular and far from constant, and the precise conditions which govern their appearance and disappearance are not completely known, apart from their relation to dissociation. In the course of induction of the change by lithium chloride it was frequently found that the first sign of alteration was an increasing roughness of the paratyphoid forms, followed later by the sudden appearance first of a few, and then still later (10th or 12th transfer for instance) of a large number of G colonies.

Microscopic characters of the G-forms.

The microscopic characters of the G forms in the present study have been found to coincide exactly with the descriptions given by Hadley and his co-workers. The morphological unit was a Gram-negative coccus of wide range of size. The largest of these were about 1.0  $\mu$  in diameter while under a magnification of x 1000 minute cocci could be seen just bordering on the limits of visibility. The cocci were variably arranged --- singly, in pairs, in quartets, in short streptoformations or in small clusters. Cover-glass impression films made directly from G-type colonies revealed elements of the same kind, with, in addition, filaments and sheath-like structures (see Fig. 6) which are probably identical with similar structures drawn attention to by Hadley.

Cytological aspects of the problem of origin of the G elements.

It appears indisputable that the G form is genetically derived from the adult or "normal" S or R forms. The complete evidence in support of this view will be summarised and discussed more critically in a later section, but meantime it is of interest to deal with those facts which indicate the manner in which the G forms actually emerge or are generated from the parent cells of the culture.

It has already been pointed out that cultures during the elaboration of the G form were observed to contain numerous rods showing various granular changes. The granules varied in size from about  $0.3\mu$  -  $1.0\mu$  and each bacterial cell might contain three or four. The appearances indicated a disintegrative change in the parent organism leading to the liberation of several coccal granules, and one can only suppose that these forms are capable of an autonomous or separate existence and of reproduction as independent organisms.

Again, similar cultures frequently showed considerable numbers of rod forms exhibiting lateral budding. In most cases the cell possessed one such bud lying close to the cell wall, but others showed in addition a secondary and smaller bud lying alongside the first. Lohnis (1921) pointed out that similar structures were first depicted by Koch in 1877 in one of the earliest microphotographs (original Fig. XVI, 5) of the anthrax bacillus. Koch termed these bodies "seitliche Sporen", and Hadley suggested, as Koch made no further reference to them, that it may be believed that he eventually became converted to the view, prevalent at the time and maintained since by the majority of bacteriologists, that these "spores" represented merely the extrusion of incidental

materials (such as protein, wax droplets or fat) from the cell.

Similar appearances have been drawn attention to and pictured by Cunningham (see Figs. 56, 57). In the present study it will be recalled that certain dissociative changes in the parent strain at the time of emergence of the original G forms led one to suspect that the G elements might arise as the result of a disintegrative change occurring in the organisms at the periphery of SR or R colonies. In this connection it is of interest that the clearest evidence of at least one mode of origin of the G forms and the G colonies was obtained by Hadley from an R colony of B. coli. "This was still an imperfect R and had been obtained from a pure S colony as a result of growth in 0.35 per cent. lithium chloride broth. A plating from the twelfth tube in the series had revealed fair R colonies, although the inner portions still contained a considerable number of S elements. Up to this point no G forms had appeared. The margins of the colonies, after five or six days of growth, became progressively more R-like, giving the typical 'cut-glass' appearance common to the R forms of many species. When this plate was 8 days old, the microscopic picture of the edge showed large spherical bodies, swollen rods and long filaments with peculiar swellings. Many of the filaments contained small granules. An attempt was made

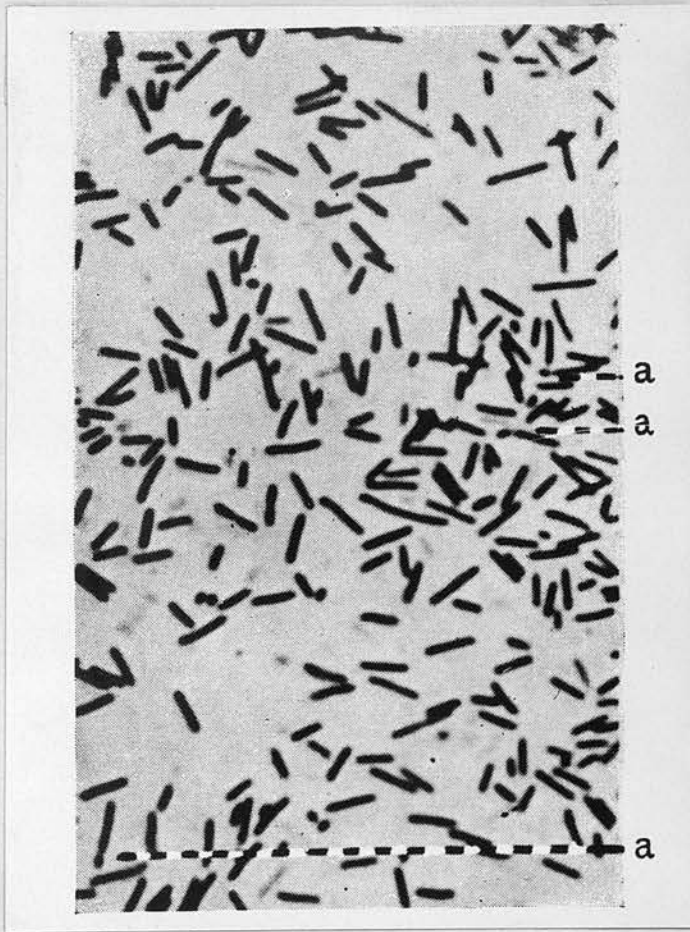


Fig. 56. Bud-like formations in  
B. saccharobutyricus.

(Cunningham).

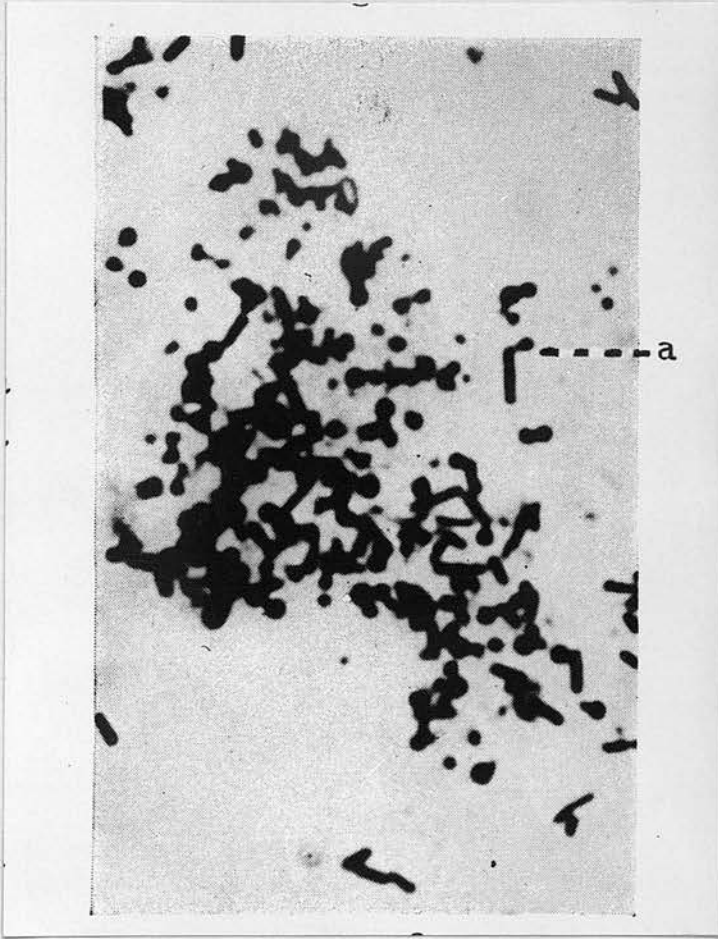


Fig. 57. Bud-like formations in  
B. saccharobutyricus.

(Cunningham).

by further plating from the edge of this colony to secure a more perfect R type.

"Accordingly, a needle was touched to the edge of the colony and the inoculum diluted in plain broth of pH 7.8. Without delay, this was spread on a plain agar plate of pH 7.4, and the plate incubated for twenty-four hours. On examination, this plate revealed, not the expected R colonies, but only G colonies and a very few GS intermediates. No S, SR, or R colonies appeared.

"When cover-glass impression films were made from the G colonies, they revealed, curiously enough, not typical G elements, but chiefly swollen rods and filaments. These, however, were unlike those of the original R colony. They scarcely took the stain and appeared like dead cells. Occasionally the rods and filaments contained minute, deeply staining granules. Similar granular bodies and cocci were scattered between the rods and filaments."

Again, in their experiments with Staphylococcus aureus, Hoffstadt and Youmans (1932) dissociated an organism, recently recovered from a human infection, in lithium chloride broth. The actual emergence of G colonies was preceded by the appearance of SR forms, and these clearly showed the development of G colonies at the periphery.

Interpretation of the physiological function of the  
G-forms.

Hadley regarded it as reasonable to believe that the generation of the G forms and associated filterable elements was related in some way to the occurrence of granular changes in bacterial cells, and he further looked on many of these granules as possessing a reproductive function. "Among these elements, we have referred to some as 'gonidia' and some as 'microgonidia'. Although we are not sure that the botanical literature indicates the existence of any qualitative difference between these two forms, it may be added that we have used these terms in the following sense: we regard the gonidia as the relatively large coccal elements that are first liberated by buddings or by 'gemmulation' from the parent cells or filaments of the culture. These may regenerate, in the course of a few generations, the original cell and culture type. On the other hand, it is also apparent that these bodies, instead of at once instituting a reversion to the original form, may continue to divide into smaller and smaller elements before the reversion finally occurs. It is indicated that some of them lie below the range of ordinary microscopic vision. It is these smallest elements that we refer to as the microgonidia. We have seen evidence of these accessory divisions

in the gonidia of the Shiga bacillus and the anthrax bacillus, and they have been reported often, in the older literature, for the cholera vibrio and some other species (Lohnis 1921)."

While this interpretation may very probably prove correct, it seems more attractive to the present writer simply to regard the emergence of the G-type as a form of "physiological variation", with inherent capabilities of reversion to the original parent type. Indeed, Hadley did not press his analogy unduly, and pointed out that further study might reveal distinct differences between the G-forms and the fungal gonidia. It is interesting in this connection that Hort (1916) described gemmation in warm slide preparations of B. dysenteriae Shiga-Kruse, B. coli communis and B. dysenteriae-Y.

Biochemical reactions of G-type cultures.

The biochemical reactions used throughout this investigation were such as are commonly employed in the routine investigation of the Gram-negative intestinal bacilli. As a rule, attention was directed to the behaviour of the organism to be tested as regards the fermentation of glucose, lactose, saccharose, dulcitol, mannitol, and xylose; the production of  $H_2S$ ; and the production of indole in peptone water.

The G strain on repeated tests over long periods of time consistently gave the reactions already referred to: acid production occurred briskly in glucose, lactose and saccharose alone, while no blackening occurred in lead acetate agar and no indole production could be detected even after many days of growth in peptone water.

These reactions further indicate the streptococcus-like nature of the G-type organism. They bear no possible resemblance to those of the parent culture, a characteristic also noted by Hadley and his co-workers, in their description of the biochemical characters of the Shiga G-type.

Heat-resistance of the G-type.

Preliminary observations made by Hadley et al. on a culture of the cholera vibrio suggested that the G forms had a higher thermal death point than the S type culture, although this was not confirmed for the G forms of B. dysenteriae Shiga. The work done in the present study has clearly indicated that, although extremely variable, the heat-resisting powers of the G form may at times be comparable with those of the most resistant bacterial spores. It has already been observed that Hoffstadt and Youmans (1932) found the thermal death point of the G form of Staphylococcus aureus to be 75 °C. as compared with 55 °C. for the normal form.

Serological reactions of the G-type.

The results obtained by Hadley in this connection seem to be identical with those in the present investigation. Most important is the finding that the anti-G serum had no action on the S or R antigens, while agglutinating well its own antigen.

On the other hand there is considerable diversity in the findings reported for different bacterial species. Hoffstadt, Youmans and Clark (1934) showed by absorption tests that the S and R organisms (of Staphylococcus aureus) contained S,

R and G agglutinogens while the G organism contained S and G and no appreciable R agglutinogens. In a later investigation (Hoffstadt and Youmans 1935) the immunological response to the living rough and gonidial variants of Staphylococcus aureus was studied in fifty-six rabbits, of which twenty were injected with the gonidial form and thirty-six with the rough, ten normal rabbits being employed as controls. Agglutinin-absorption methods were used. Serum produced with living and heat-killed rough and smooth strains of the organism showed cross-agglutination for the living organisms, while that produced by immunisation with the living gonidial strain showed agglutination for all three forms, although the serum produced against heat-killed gonidial organisms only cross-agglutinated for the gonidial form. Hoffstadt and Youmans also determined that immunisation with the gonidial (and rough) forms of Staphylococcus aureus afforded no protection against infection with the smooth virulent form of the same organism, and that the gonidial forms contained a heat-labile fraction in common with the rough and smooth forms.

In the second paper by Swingle (1935) --- also dealing with small colony variants of Staphylococcus aureus --- some variation was observed in the behaviour of different strains, but variants

of three of the four normal strains studied were antigenically similar to their source strains as judged by agglutination reactions, and the same variants showed cross-agglutination with each other. Sera prepared against the small colony strains likewise agglutinated the normal organisms, although the titres were rather low against both the variants and the normal bacteria. In the work of Fabian and McCullough (1934) already referred to regarding dissociative processes in yeasts, limited serological studies indicated a closer relationship between the R and G forms than between the S form on the one hand and the R and G on the other.

It is clear that there is no uniformity as to the nature of the serological changes in the S----->R----->G dissociation, and it seems indeed as though an organism in some cases may assume the morphological and physiological characters of the G form without undergoing any major change in agglutinogenic structure. In other cases however, the change may be so marked as to render the G form serologically independent of the parent type. In this connection it should again be noted that the agglutination results obtained in this investigation on B. paratyphosus B agree almost completely with those recorded by Hadley in the case of B. dysenteriae Shiga.

Toxicity and virulence.

All the evidence to date confirms Hadley's experience that live G-type cultures can be inoculated intravenously in considerable amount in rabbits without the appearance of unfavourable symptoms. This has also been the uniform experience of other workers, and suggests that the G form is devoid of toxicity or virulence. In the case of the G forms of B. typhosus, however, Hadley and Carapetian (1933) obtained some evidence to show that intraperitoneal inoculation in guinea-pigs may induce a mild transmissible disease simulating that described by Friedberger and his co-workers in 1917 for the invisible form of B. typhosus.

Filtration.

As has already been indicated, the present study was not prosecuted from the point of view of the filterability of the G elements, although sufficient work was done to indicate that these forms (or elements accompanying them) are, in Hadley's words, "readily and invariably filterable and that they can be recovered from the filtrates in nearly all instances by the employment of a suitable cultivation technique, although no trace of them may be found by ordinary methods."

In Hadley's extensive filtration experiments he employed Berkefeld candles of grades V, N and W, mostly new, although the age of the candle seemed to make no difference to the results obtained. In later tests most of his candles were tested by air pressure and no N or W candles were employed unless they could withstand an air pressure of 9 lbs; under these circumstances the ordinary S and R forms of the Shiga bacillus did not pass the candle in any case. Fractional filtration was employed in many instances. The amounts filtered were usually between 20 and 35 c.c., and generally under a negative pressure which however never exceeded 300 mg. Hg.

For filtration purposes the G-type cultures, prepared from single colonies by "fishing" with glass threads, were grown in beef infusion broth of pH 7.6 for from 24 to 48 hours at 37 C.. When such G cultures, which ordinarily showed only a faint clouding, were filtered through Berkefeld candles of any grade, some of the elements in the culture almost invariably passed the candles. The presence of these filterable bodies was demonstrated in three ways:

(1) by growth in the original filtrate after incubation in unsealed tubes; (2) by growth in sealed ampoules after a period of from several months to two years or more, and (3) by serial plate cultivation of the fresh filtrates on plain infusion agar, litmus lactose agar or blood agar. (For details of the conditions laid down by Hadley for filterability, see Hadley, Delves and Klimek, 1931, pp. 132-133.).

Hadley's filtration results have received most striking and literal confirmation in the course of the present work, and the sequence of events may best be described in his own words: "When growth first appeared in the samples of tubed filtrates, it was invariably long delayed --- sometimes for weeks or months --- and seldom gave a distinct clouding of the medium. At the end of several months there was often an opalescence and usually a viscogranular sediment. From such tubes, whether showing opalescence or

sediments, or remaining clear, the G type culture could eventually be recovered by the serial plating method .....In the case of sealed filtrates two or more years old, the G colonies often arose on the first plate after an incubation at 37<sup>0</sup> C. for from six to ten days.....

"The seeding of from 5 to 10 drops of fresh filtrate on a sterile agar plate usually resulted, after two or three days' incubation, in what seemed to be a sterile plate; only a delicate 'film' could be discerned. Even under microscopic examination nothing that resembled colony structure was revealed. When this film was washed up in from 10 to 15 drops of sterile broth and transferred to a second plate, this plate also remained apparently sterile, although, like the first, it presented a film-like surface. On the third, fourth, fifth or later plate, however, similarly inoculated from the plate just preceding it in the series, it was possible to detect by a hand lens (3.5x to 10x), a no. 3 objective or even by the unaided eye the very minute and delicate colonies representing the G type culture, apparently taking form from the film-like debris. Continued plate transfers in series at frequent intervals served to maintain the culture in this form for many generations. This is essentially the method first employed by Hauduroy (1927a) and, as in the cases described by him, the continued plating

rendered the growth more stable on the solid medium."

Mudd pointed out that the observation of a colony or two on a plate seeded with a considerable amount of filtrate is not, in itself, a striking or significant phenomenon. On the other hand, as Hadley replied, if a filtrate presenting the optical appearance of sterility reveals hundreds or even thousands of filterable and cultivable bodies for every drop, one has better reason to suspect in the species concerned the existence of elements that could appropriately be described as "filterable forms". This last state of affairs was frequently met with on plating filtrates from V candles, when seeding the plates with 5 drops of the filtrate gave hundreds or even thousands of colonies visible to the unaided eye. "In one case, a V filtrate was collected in seven fractions, taken at intervals of thirty seconds, and 0.5 c.c. of each fraction was seeded on an agar plate. After twenty-four hours' incubation, plate 1 gave 20 visible colonies, plate 2 gave 200, plate 3 gave none, plate 4 gave 10 and plate 5 gave none. After thirty-six hours' incubation however, plates 3 and 5, previously negative, each showed more than 100 colonies, and the number was increased proportionately on the other plates. There were occasional instances in which the seeding of one loop of a fresh V or N filtrate yielded, after from thirty-six to forty-eight hours, many hundreds of G

colonies." Such results indicate that the numbers of elements traversing the filter are sufficiently large to possess definite significance.

In the present work the original G strain was first obtained in a perfectly pure condition after filtration, apparently by mechanical separation of the G forms from the abnormal bacillary forms present in the dwarf transitional colonies already described (vide supra). But as Hadley pointed out, the filterable forms are not 'produced' by filtration, since in the experiments which he described the variants were first present and demonstrated by plating methods in the original culture submitted to the filtration process. The same statement holds good for other G strains recovered in the present work from B. paratyphosus B transfers in lithium chloride broth.

Hadley also observed a significant relationship between the rapidity with which the G type culture developed and the grade of candle employed for the test. In general it was observed that when V candles were employed, the colonies appeared earlier in the series, although there were exceptions; and that when N or W candles were used, the colonies were more likely to appear later. Thus in nine tests with V filtrates the G colonies appeared on the first plate in seven instances. Of twenty-seven N filtrates, five gave G

colonies on the first plate and three on the eighth plate, but they gave G colonies first most commonly on the second, third, fourth and fifth plates, the largest number giving the colonies first on the fourth. Of five W filtrates, all registered the first G colonies on the third, fourth or fifth plate.

Gradual development of G-type cultures in stored filtrates.

Hadley drew attention to the fact that when a culture of the Shiga bacillus undergoes lysis under the influence of bacteriophage, and is then filtered through a Berkefeld or Chamberland candle and the filtrate set aside for some weeks or months, an opalescence or sometimes a faint clouding appears or in other cases the formation of a slight sediment. These phenomena are due to the generation of a new form of the original culture. The new organisms are usually small coccal forms more resistant or even extremely resistant, as d'Herelle showed, to the action of the bacteriophage. In the course of time however they may become re-transformed into the original culture type. As has already been pointed out, typical G colonies were observed in the course of the present investigation when plates were seeded from

such bacteriophage filtrates.

In view of these observations relating to the development of a secondary culture growth from bacteriophage filtrates, it was found of interest to study the behaviour of the G type culture filtrates with the lapse of time. In several filtration tests involving mainly N and W candles, Hadley observed that certain filtrates which failed to yield G colonies on the first plate of the series when the filtrate was fresh gave a fair growth of G colonies after the filtrates had stood at 37° C., or even at room temperature, for a longer period. In other words, the elements present in the fresh filtrates seemed to be non-cultivable, while elements present in filtrates six or more days old were more easily cultivable on agar plates. These observations seemed to indicate that the living elements present in the stored filtrates become more cultivable with the lapse of time. Other tests showed that the appearance of the G colonies on plates seeded with the filtrates of G cultures was dependent not only on the number of plates carried in the series, but also on the age of the filtrate when the first plate was inoculated. In other words, the G colonies could be obtained either by carrying forward from plate to plate the washings of each in turn or by waiting for the filtrate to age a sufficient time. This phenomenon was found by Hadley not to be limited to the G forms of the Shiga

bacillus: it has also been observed repeatedly in the present investigation and was also recorded by Hadley in the case of B. paratyphosus B, as well as with the G forms of B. typhosus, B. typhi-murium and B. enteritidis.

"These observations and others that might be reported reveal the fact that the filterable elements present in the freshly derived filtrates of G type cultures of various bacterial species seldom exist in a form that is visibly cultivable on agar plates under ordinary conditions of growth until many days have passed. The manifestation of this 'latent' period varies with the grade of candle and probably with the state of the G type culture at the time of filtration. It has been our experience that this period is, as a rule, from six to twelve days; but it may be much longer, as we have shown in the case of the filtrate of a diphtheria G type culture. With the passage of this 'latent' period, the filterable elements seem to have entered a stage of development in which they are able, not merely to survive, but to register directly on the plates by the formation of the G colonies. Before this time has arrived, several serial cultivations on agar are necessary to reveal their presence. It should also be recognised that these positive results in plate cultivations succeed in the case of filtrates that, at the time of first attempted

cultivation, may reveal no sign of growth, either by sediment, opalescence or clouding.....It might be considered possible that serial cultivation of the filterable elements on agar plates, in order to raise them to the estate of the visible G type culture, is really unnecessary; that, on the contrary, if the first plate on which the fresh filtrate is seeded were kept for a sufficient time, the G colonies would eventually appear. While the "ripening" of the filtrates themselves over a period of days or weeks or months encourages the development of the filterable elements, it has not been commonly observed that continued ageing on the original plate, containing ample medium and protected against drying, accomplishes this end. We have some evidence that this circumstance involves in part the question of ample moisture on the plate surface."

Constancy of filterability of G-type cultures.

One of the most striking features of the present study has been the ease with which the use of Hadley's methods has led to results fully confirming his own original findings; and this applies no less to the apparent constancy of filterability of the G elements. This raises the question whether filterability is a perfectly constant feature of such cultures, and

Hadley devoted special attention to this problem. In actual experiments a negative filtrate was never obtained in twenty-two filtrations, whether performed through V, N or W candles. In every instance, distinct G colonies came into evidence on one of the plates, most commonly on the fourth or fifth of the series. Further examination of these colonies showed them to correspond in all respects with the original Shiga G type culture. Hadley therefore concluded that G type cultures are invariably filterable, that is, the same G strain can always be recovered from the filtrates; but he was led to emphasise the fact that if the demonstration of the passage of the filterable bodies through the candles had rested on the discovery of the G colonies on the first, or second, plate, negative results would have been obtained in sixteen of the twenty-two filtrations. If the demonstration had rested on the first plate alone, negative results would have been assigned to eighteen of the twenty-two filtrations.

Relation to other work on filtration.

In most of the reported work on bacterial filterability it is the rule to find that evidence of growth has been sought for from filtrates of a "normal" culture. On the other hand Hadley

emphasised the fact that the majority of such cultures are almost certainly non-filterable in the usual sense. His own contribution to the problem consisted in the demonstration that filterability is associated with the presence in the culture of a specific variant, and that this might be produced by instigating a dissociative reaction in the mother culture. After isolation they were demonstrated to be filterable, that is, filtrates were shown to be capable, when given adequate growth facilities, of yielding cultures comparable in every respect with the G type cultures originally employed for the filtration tests.

Work of this kind is nowhere more unsatisfactory than in the case of the tubercle bacillus, but it is of interest that certain work reported by Mellon and Jost and by Panek and Zakharoff seems to indicate that a similar mechanism may govern the occasional filterability of the tubercle bacillus. Mellon used for his filtration tests a strain in which the individual organisms were characterised by the presence of "granules", and Panek and Zakharoff have shown that the granules are cultivable independently of the rod forms. The granular stage thus seems to bear comparison with what Hadley termed the G type culture. Hadley laid stress on the fact that the filterable form of the Shiga bacillus was not produced by filtration but was first demonstrated

as associated with a recognisable variation in the parent culture and only subsequently shown to be filterable. In the present investigation the first isolation of G forms, agreeing with Hadley's description in every particular, was from filtrates of a broth culture which prior to filtration gave large numbers of dwarf transitional colonies on plating. Apparently here a series of coccoid elements, graded in size, was associated in these dwarf colonies with disintegrating bacillary forms, and the filtration apparently acted by mechanically separating the latter and allowing the G forms to be recovered in a pure condition. Later, as has been shown, G strains were also recovered in the course of variation induced by passage of the parent culture through lithium chloride broth, without recourse to filtration.

On the other hand, Hadley pointed out that the process of filtration, interposed in the development of the Shiga G type culture, modified this culture in at least two important respects. First, it transformed a culture easily cultivable on agar into one that, for a variable time, was visibly non-cultivable either on agar or in broth. This he ascribed to a separation of elements less readily cultivated from those more readily cultivated, and it must also be due in part to the separation of the smallest elements of the G type culture from those

larger elements which are found in, and characteristic of, a developed G type colony. Visible cultivability from filtrate, when it appears, is apparently associated with an increase to visible size of these filter-passing elements. Secondly, an interposed filtration tended to stabilise the G form against a rapid re-transformation into or towards the original S culture type. It was observed that G colonies in a strain which had not undergone a single filtration showed a definite tendency to fairly rapid reversion to the "normal" culture. On the other hand even one filtration through an N or W candle resulted in a G type culture which underwent reversion only by much slower steps and after a longer interval. From these circumstances Hadley suggested that the G type in its cultivable form represented a stage of transition bridging the gap between the primitive filterable virus form and the original culture type. These facts will be dealt with later in the succeeding section relating to reversion. Hadley in all performed over 200 filtrations on material which showed, on culture prior to filtration, the presence of G colonies in all but two cases. From the filtrates by the methods described he recovered an identical form, in the pure state, in all but three instances.

Reversion of G-type cultures.

A later section will be devoted to a discussion of the nature of the biological relationship which exists between the G form and the original culture, particularly whether the association be a symbiosis between two essentially unrelated forms or whether the two forms are genetically related. The latter possibility would obviously receive practically conclusive support could it be shown that the G form, apparently derived from the so-called "parent" culture, may undergo mass reversion under suitable circumstances, that is, whether transformation of one form into the other is reversible. It may be stated at the outset that this is the only part of this problem where the results of the present study do not afford almost complete confirmation of Hadley's findings. In over three hundred transfers of G type cultures in the present work, no indication of mass reversion was ever observed. On the other hand, Hadley obtained complete mass reversion in the case of seven of his G strains studied, in five instances to the S type Shiga and in two to the R form. That reversion might occur was first indicated by the course of events observed in Shiga cultures in which the G type had been induced by the application of a suitable dissociating agent; several examples were found in which normal S or SR forms were recovered from the broth transfers after one or more agar

plates had revealed G colonies only. In these cases the "normal" forms re-appeared after a longer or shorter absence, but obviously such cases were not conclusive proof of reversion, particularly since the sequence S----->S plus G----->G----->S occurred within a relatively short time and also because other alternative explanations are equally likely.

Accordingly, reversion from G to S or R forms was looked for in pure line G strains. The first pure line G strain to manifest complete reversion was one derived from an R type Shiga culture. "In this case, the reversion to the R type took place very slowly, being accomplished by about sixty serial transfers at twenty-four hour intervals through plain infusion broth. Frequently, for a week or more of daily passages, it was impossible to detect any change in colony form. Over the entire period, however, it was observed that there occurred a gradual increase in the size of the G colonies from 0.2 mm. or less to about 2 mm. (the average size of the S colonies) or to from 3 to 5 mm. (the average size of the R colonies). This slow change was accompanied by a loss of the delicate structure and of translucency and by an increase in opacity..... It was at about the sixtieth passage when the G form seemed to have reverted completely and to have taken on the biochemical, and at least some of the serologic, characters of the original type. It had also again

become sensitive to a mixed Shiga bacteriophage." Another pure line G strain, derived from an S type, gradually reverted to a typical Shiga S type culture after about fifty-three daily passages through plain infusion broth. A third G type culture, which Hadley recovered from a Shiga culture dissociated by pancreatin, reverted to a typical S type culture in rather a different manner. After a few preliminary transfers, this G culture was stored in a sealed ampoule at room temperature for four months, when the cultural characters were apparently unchanged. Thereafter the culture was transferred in broth at intervals of two weeks, each successive transfer being made from the largest colony developing from a plating of the preceding transfer on agar. By repeated selection of this kind the colonies in about four months time had nearly attained the diameter of the S type Shiga colonies (2 mm.) and resembled them in other respects. After five months, no further differences could be noticed, and the culture now agglutinated in Shiga S type serum. It resembled B. dysenteriae Shiga biochemically, was toxic for rabbits, and had again become susceptible to the Shiga bacteriophage.

Although reversion of this kind has never been observed in the work now being reported, there is no reason to deny that it may occur as Hadley describes. The phenomenon is apparently far from invariable in

occurrence, and Hadley referred to pure G strains kept for one to two years without altering in any of their characteristics. Further, not all deliberate attempts to induce reversion proved successful, but, on the contrary, certain strains manifested a condition of apparently permanent stability. Even when conditions for reversion are suitable (as can only be shown by eventual success) the process appears invariably slow. Further, in the present work most of the attempts to demonstrate reversion were carried out with a G strain originally recovered from a Berkefeld filtrate, and, as has been stated, Hadley showed that the selection of certain elements determined by the filtration process seems to exert a stabilising influence on the G forms and that filtration at intervals may actually be employed to hold a G culture true to type.

In view of the relative importance of this aspect of the matter, specially devised experiments were carried out in the course of the present study in order to accelerate reversion if possible. All of these failed in their primary purpose, although some yielded side-results of interest.

In the first place, the growth of G type cultures was studied in larger volumes of medium than those employed in the regular serial transfers. Penfold (1913) found that reversion to gas-production

took place more rapidly in a large two- to three-litre flask than in a smaller volume, and argued that this was due to the large amount of growth unrestrained by crowding and by-products, since this rapid growth led to the maximum variation and a larger number of variants for selection to act on. In the present study flasks of broth containing 200-250 c.c. were used, but over prolonged periods of observation by plating no indication of reversion was obtained. Parallel experiments were carried out in similar volumes of broth containing 10 per cent. of an anti-G agglutinating serum; in this case a considerable amount of growth with sedimentation took place, but the only change detected on repeated plating was the production of a rough change in the contour of the G colonies, which again showed no tendency to revert. It is of interest that Hadley's G strain III (which subsequently reverted to the original culture type) showed no trace of reversion during ten serial transfers at somewhat irregular intervals through 10 per cent. anti-S Shiga immune serum broth.

Secondly, G type cultures were maintained and subcultures made in fluid media other than plain nutrient broth. As indicated above, the growth obtained in ordinary broth never showed more than slight turbidity and frequently gave indications

of spontaneous autolysis. It was accordingly felt that the supply of a richer pabulum might increase the possibility of successful mass reversion, secondary either to increased growth-rate due to an ampler supply of non-specific foodstuff, or conceivably to the presence of more essential or specific substances in these richer media but absent from ordinary broth. Various media were employed at different times for this purpose. Phosphate broth, as used for the cultivation of streptococci, was found to give remarkably good growth of the G-type culture, but again no sign of reversion appeared. Secondly, culture was carried out in liver-broth for some twenty serial transfers; growth was variable in amount, and although a new variant was recovered from this experiment (strain G 207L, above), no tendency to mass reversion was observed on repeated platings on agar. Further experiments were carried out by growing the G-type and certain derived strains in broth containing 5 per cent. of a killed suspension of B. paratyphosus B, but again with negative results.

In the third place, although no bacteriophage activity of any kind has ever been observed in the G cultures studied on repeated test, it was considered possible that the presence of bacteriophage or some other factor in the G cultures might conceivably inhibit any tendency to reversion in the early stages by destroying the bacilli, by lysis or other means, on

their emergence. Attention has been drawn to the view held by Kendall (see Kendall and Walker 1933) that bacteriophage filtrates frequently contain filterable elements derived from the organisms on which the bacteriophage was propagated prior to separation by filtration. Kendall suggested that these forms remained in the filterable stage on account of the high concentration of bacteriophage with which they were surrounded, and he furthermore claimed to have demonstrated their reversion to the original organism by inoculating numbers of broth tubes with amounts of bacteriophage filtrate subjected to serial dilution over a range exceeding the end-point of demonstrable bacteriophage activity. As well as inhibiting the lytic principle by dilution, he also deviated any bacteriophage present by the addition of killed suspensions of the homologous organism. In Kendall's hands the use of this ingenious though simple procedure resulted in the recovery of adult 'normal' homologous bacilli, and he rightly attached great significance to the fact that very frequently these first appeared in the tube which corresponded to the extinction of the bacteriophage elements by dilution. Similar experiments were carried out in the course of the present work by inoculating broth tubes with serially diluted inocula of a young G type culture, and uniform additions of killed paratyphoid bacilli

were also made to the broth tubes on the same principle as in Kendall's work. In all experiments save one, quite negative results were obtained, indicating that the lack of a reversionary tendency in the G type culture could not be attributed to the presence of any inhibitory factor of the nature of bacteriophage. In the single positive experiment already described, a Gram-negative bacillus (SS2) was recovered which showed obvious biochemical resemblances to the other variants encountered in this work, although its precise derivation from the G-type culture could not be proved.

Deliberate attempts to influence reversion by animal passage were not employed in the present work in view of the notorious difficulty of ensuring that cultures recovered from inoculated animals are in fact descended from the inoculum. Experiments were however carried out by taking repeated daily blood-cultures from rabbits previously inoculated intravenously with live broth cultures of the G organism; and the organs of animals similarly inoculated for the production of agglutinating antisera were cultured immediately after the death of the animal. Again the results gave no hint of the occurrence of reversion.

Thus, while the experience relating to reversion gained in the present investigation is less

complete than that of Hadley in the sense that mass reversion itself was not encountered over a considerable period of observation, no evidence was found which might throw doubt on the reality of the reversion process observed by the American workers. Indeed, reversion in some of Hadley's strains was never observed and in certain others was greatly delayed by the selective action of filtration. As will be seen later, there is no theoretical reason why such a variant in certain conditions should not manifest an appearance, or even the reality, of complete stability.

Hadley laid emphasis on the extremely slow and gradual nature of the process of re-transformation compared with the relatively sudden generation of the G colonies at the beginning, when they first arise from the S or the R cells. While their first origin is what might be called 'explosive' or manifestly discontinuous, their return is likely to be a matter of weeks or months. Hadley paid little attention to the problem of the biological changes occurring in the reversionary process but indicated that the transition occurred by 'steps' and that the cells characteristic of these intermediate stages would not be recognised as members of the Shiga species. Again he had no evidence as to the actual point in the gradual change from the G to the mother

culture at which filterability was lost, or at what point other characters, such as susceptibility to bacteriophage, were regained. He apparently had evidence leading him to suspect that the intermediate stages represented a gamut of biochemical and serological characteristics, but gave no actual details. Although the present study can give no complete picture of mass reversion, it is in this matter, the question of the biological nature of the re-transformation, that it may afford more detailed information than that of Hadley.

Although complete reversion was not attained, evidence accumulated to show that a G-type culture in course of serial propagation manifests a definite tendency to vary towards a bacillary type. As described in the first section above, discontinuous variants appeared from time to time, and these when isolated and maintained in pure culture as individual sub-strains showed in turn the phenomena of stability, reversion, or a similar variability. Certain points must be made quite clear with regard to these variants. First, in no case did the variation observed affect the whole body of the culture. The variants were mainly detected by the appearance of colony variations on plates inoculated from the serial transfers in broth. In some cases they affected only a small proportion of the cells in the varying culture, as evidenced by the low ratio of variant to normal

colonies, while in other cases the new variation was manifest on a much larger scale although not involving the whole body of the culture. Secondly, the observed variations, although perfectly definite and affecting individual characteristics in a sharp all-or-none fashion, remained within a limited range. Thus they were all anaerogenic and insensitive to a bacteriophage active for B. paratyphosus B. Biochemical and serological classification gave definite support to the suggestion, if not an actual proof, that they are genetically derived from the G strain and accordingly related to one another. The bacillary forms must obviously be regarded as extremely dedifferentiated in their characters as compared with the paratyphoid bacillus, but it is not outside possibility to postulate that they represent some of the stages through which a G-type culture passes, as Hadley suspected, in the course of a step-like transformation to the fully differentiated form.

Table V shows the main features characteristic of the G strains I and II --- the latter obtained by the action of lithium chloride --- and of several derivants of the former, with, in addition, strains Auto B and SS2. Study of this table shows that these organisms may readily be classified in certain groups as follows: Group 1 (G strain I, G 41, G/158, and Auto B) indistinguishable from one another by the

reactions tested; Group 2 (G 74F and G 41/34/24) indistinguishable from each other by the reactions tested although different from the organisms of Group 1; Group 3 (G 74/48, G 41/34/24/69, G strain II), indistinguishable from one another but different from the organisms of Groups 1 and 2. Several other features of interest appear from the table. Thus, strain G 74/30 differs from G strain I merely in the absence of lactose fermentation, and G 74/30/73 in turn differs from G 74/30 merely in morphology and mannite fermentation. Again, strains G 41/34/24/69 and G 74/48 (identical) were derived respectively from strains G 41/34/24 and G 74F (identical) through a simple loss of motility. Thirdly, it is of interest that, while they are distinct, the sum of the characters of strains G 74/30/73 and SS2 is equal to the sum of the characters already possessed by either strain G 41/34/24 or G 74F, except for the absence of lactose fermentation. Fourthly, the biochemical behaviour of G strain II is duplicated by that of strains 74/48 and 41/34/24/69, although the latter organisms were Gram-negative non-motile bacilli and the former a Gram-negative coccus. All these facts tend to indicate the degrees of mutual relationship existing between the G strain and its derivants, and also between the derivants themselves. Attention may also be drawn to the indifferent biochemical behaviour shown by strain G 207L, a feature also found in the

case of a later strain, G/252. It is of interest that a similar inactivity was observed in organisms cultivated from slow-growing dwarf-colony variants of B. paratyphosus B, a colony type which has also been recognised by other workers (see above), although it is quite distinct from the true G phase.

Additional confirmatory evidence of these degrees of relationship was gained from a serological study. It should be mentioned in passing that the serological examination of these strains proved to be technically more difficult than was anticipated, mainly due to the presence or development of excessive roughness. This was frequently accompanied by poor antigenicity and, in the tests themselves, difficulty in preparing satisfactory suspensions. Again, in the case of the coccal forms (G I, G 41, G 74/30, Auto B), difficulties occurred of the type met with in streptococcal agglutination reactions. Most satisfactory results were obtained by using live organisms centrifugalised from phosphate broth cultures and re-suspended in sufficient saline to yield a suitable opacity, and the tests themselves were read after 1 hour at 55° C. Experience gained in the present work also indicated that the agglutinins evoked in response to immunisation with these variants were more labile, and showed a faster rate of disappearance on storage,

than is the case with agglutinins induced by immunisation with "mature" organisms such as are ordinarily used for the preparation of high-titre diagnostic agglutinating antisera. It was thus observed that various batches of sera which were very definitely active in reactions performed within a few weeks of the preparation of the serum, (giving complete agglutination at dilutions of the order of 1 in 6,000), showed only a trace or in some cases no demonstrable agglutinating activity after storage, in the ice-box, for nine to twelve months. But by the use of reasonably fresh antisera it was possible in most cases to secure indubitably clear-cut reactions in the majority of tests. Table indicates the most important of these results, and all of these reactions were obtained on numerous occasions. From these data there is apparent identity (1) of strains G 74/48 and G 41/34/24/69; (2) of strains G 41 and G 74/30; and (3) of strains G I and Auto B, results which are in harmony with the similarity or identity which these organisms exhibit as regards biochemical activity. The last case, identity between strains G I and Auto B, was also proved by agglutinin-absorption tests.

To conclude discussion of this aspect thus far, the sum of these results and of those obtained by Hadley indicates that the G form, which arises sudden-

T A B L E V I I

Sera prepared against strains..... G 74/30 G 74/48 G Auto B.  
 Strains. 41/34/24/69 SS-2 G/158 G 41 G 74/30 G 74/48 G Auto B.

41/34/24/69.	1/6400	0	0	0	0	1/800	0	0
SS-2	0	1/3200	0	0	0	0	0	0
G/158	0	0	1/6400	0	1/800	0	1/3200	1/1600
G 41	0	1/800	1/1600	1/1600	1/1600	0	1/1600	1/1600
G 74/30	0	1/3200	1/6400	1/3200	1/6400	0	1/1600	1/6400
G 74/48	1/3200	0	0	0	0	1/1600	0	0
G	0	0	1/3200	1/3200	1/3200	0	1/3200	1/1600
Auto B.	0	0	1/1280	1/1280	1/1280	0	1/1280	1/1280

ly from the parent culture, in some cases may gradually revert to the latter by way of certain comparatively de-differentiated (e.g., anaerogenic) bacillary forms, the characters of which may be studied by isolating the organisms in pure strains by artificial selection. The biological nature of this re-transformation, and particularly whether it is to be regarded as a physiological cyclical change, will be discussed in later sections.

Nature of the G type in relation to the parent culture.

At this stage of the discussion it becomes necessary to review from a critical aspect any justification which exists for regarding the G form as a true genetic derivant of the parent organism, in this case B. paratyphosus B. In brief it will be found that while most of the evidence in favour of such a thesis is presumptive, the total is not inconsiderable and becomes more than impressive if the possibility of reversion be admitted in addition. Theoretically the truth of the suspected relationship will only become positive when the G type is generated from a single cell of B. paratyphosus B and when in turn a pure culture derived from a single cell of the G form is found to undergo mass reversion to the parent type. Short of this ideal experiment, a subject to be discussed later, the circumstantial evidence of relationship may now be summarised.

The first possible objection to the suggested interpretation, and perhaps the simplest, is to regard the G organism as a contaminant occurring in a culture originally pure. The likelihood of this explanation is reduced, although not eliminated, by the fact that the G type, if regarded as an entity quite independent of the alleged "parent" culture,

represents a species hitherto quite undescribed. As is seen from the details already described, it is a Gram-negative coccus of variable size whose slow growth-rate results in a characteristic minute colony and the appearance of only minimal turbidity in ordinary nutrient broth; while typically though variably heat-resistant and not inhibited by a medium containing bile-salts, it gives active green-production when grown on boiled blood-agar; and it can be shown to be constantly filterable by the recovery of the same organism by suitable methods from Berkefeld filtrates of broth cultures.

Secondly, any probability of its existence as an independent contaminating species is further reduced by the circumstance that, as well as appearing spontaneously and intermittently, it shows an apparent dependence on variation processes in the mother culture and can be produced experimentally under the influence of lithium chloride, bacteriophage and other dissociating agents. Further, its appearance under these latter circumstances apparently bears a definite relation to the time of action of the dissociating agent: thus Hadley found that the G types do not make their appearance until a certain moment has arrived (usually the sixth to twelfth tube in the series), and then they appear suddenly, and in considerable numbers, an observation which has been strikingly confirmed in

the present work, particularly in the emergence of strain G II under the influence of 0.5 per cent. lithium chloride. The spontaneous appearance of the G forms in a dissociating laboratory culture was observed to occur infrequently and irregularly during a period of over three years, with intervening periods of many weeks during which no trace of the G type could be detected by the ordinary cultural methods employed.

A second possibility, also discussed by Hadley, is that the G type culture, though not a laboratory contaminant in the ordinary sense, may represent a distinct biological entity living in symbiotic relationship with the so-called parent culture. The relation between the bacteriophage and the resistant organisms in its substrate was visualised by d'Herelle and Hauduroy as an analogous type of symbiosis, and more recently Klieneberger (1935) has described the natural occurrence of pleuropneumonia-like organisms in apparent symbiosis with Streptobacillus moniliformis and other bacteria.

The biological characters of the G type are manifestly unique, but as they include certain features rather suggestive of a possible affinity with the faecal streptococci, the entirely reasonable suggestion was made that the G type derived from intestinal pathogens might represent a bowel-

inhabiting organism which had existed in symbiosis with the pathogen from the time of its primary isolation from the faeces. This argument may be dismissed at once in the present case, since the Tidy strain of Salmonella paratyphi B (Schottmuller) was originally isolated by blood-culture (King George Hospital, London, 1915; see Catalogue of the National Type Culture Collection). This single circumstance greatly reduces the possibility of the existence of any such symbiotic relationship in the present case. In addition to this general argument against symbiosis, there is another which must be given due weight, namely, that repeated colony selections completely failed to purify the strain.

Hadley acknowledged that it might be regarded as unfortunate for his claims that the G forms of most of the cultures studied in his laboratory resembled one another extremely closely in regard to morphology and colony structure, since this circumstance might seem to afford evidence for a contrary hypothesis that the G form represents a "universal contaminant". But in discussing this he pointed out that many species of micrococci are alike morphologically and that, in addition, the "seed forms" of fungi often resemble each other much more closely than the adult forms, while in general, many diverse forms of life meet on a common basis in the embryo and even more so in the seed. Apart

however from these theoretical parallels and deductions from comparative biology, Hadley was able to show that while the G type cultures of the Shiga bacillus, the typhoid bacillus, the paratyphoid bacillus, B. enteritidis and other related organisms might possess closely similar features, the G forms of a remote species (B. anthracis) possessed individual characteristics sufficient to throw instant doubt on the suggestion that the G type represents some form of universally occurring contaminant.

Among variants of the anthrax bacillus studied by Nungester and Parker in Hadley's laboratory, one was found, arising commonly from a smooth type, which Nungester termed the "midget" colony. The "midget" forms were found to be filterable, giving rise to minute bodies which easily passed the Berkefeld N and W candles. On seeding the fresh filtrates to agar plates, no growth appeared earlier than the third plate in the series, but there then appeared minute colonies which seemed to resemble those seen in the Shiga cultures. A definite difference was however observed, that these colonies showed a distinct and characteristic morphology in keeping with the morphology of the anthrax colony. This observation Hadley regarded as sufficient at least to suggest that, although the G colonies and cultures of closely related species appeared practically

identical, "the analogous forms of distantly related species will be found to possess their own characteristic features which are readily distinguishable, one from the other."

Bearing these facts and criticisms in mind, one of the most striking features of the work has been the almost perfect parallelism between Hadley's recorded data and the observations made in the course of the present research. In view of the considerable number of species in which the emergence of the G type has now been observed, there seems little doubt that the phenomenon may quite readily prove to be one of general significance in bacteriology as a whole.

There is yet a further point, of great theoretical interest, which indicates with a considerable degree of certainty the real genetic derivation of the G type from the normal form. Mention has already been made of the occurrence under certain circumstances of independent secondary lactose-fermenting colonies within the larger colonies of the paratyphoid culture. This phenomenon was studied particularly in the case of (a) a broth culture of B. paratyphosus B modified by heating at 55<sup>o</sup> C. for 30 minutes, and (b) cultures of B. paratyphosus B in 0.5 per cent lithium chloride broth. While numerous attempts to separate the

papilla organism failed, in certain cases each secondary colony showed a striking morphological resemblance to the isolated G colony, and in the case of the lithium chloride cultures the phenomenon was noted particularly in 4- to 5-day-old SR or R colonies shortly before discrete G colonies appeared in large numbers on plating. The same interpretation was given this phenomenon by Hadley, in whose laboratory it was accidentally and independently observed by Bailey in the case of a pure line S type culture of B. typhosus being passed at daily intervals through plain infusion broth. The implication seems evident that the S or the R cells at a certain moment in their development in certain circumstances are able to generate or to become transformed into the cells characteristic of the G type. As Hadley showed, this does not appear in every culture and it may be necessary to wait for several tube-generations before the critical moment of transformation arrives. The biological interpretation of secondary colony formation will be treated in detail in another place, but for the present it will suffice to stress the fact, which most authors, including Arkwright (1930) acknowledge, that the appearance of papillae usually indicates the true generation of a variant.

A further circumstance has still to be alluded to which affords strong additional evidence

in support of the hypothesis that the G form represents a true genetic derivant. It will be recalled that the original G strain studied in the present work did not appear spontaneously in the pure state in the form of typical discrete colonies, but in the beginning was only found in association with dwarf colonies containing degenerate bacillary forms in addition. From these it was subsequently separated by filtration. Both the external features of these dwarf colonies and the nature of the bacilli they contained --- these giving in many cases the reactions characteristic of B. paratyphosus B --- afford certain proof of their true variant-relationship to the normal S and SR colonies which accompanied them on the plates. These facts in turn indicate most strongly that the coccal G forms occurring in these dwarf colonies must be regarded as emerging from the granular and disintegrating bacilli observed in and characteristic of the smaller colonies. Since the actual relationship of the two colony types is not to be seriously doubted, these facts supply yet another piece of independent evidence, and in this case actually indicate the nature of the cytological changes which are involved in the generation of coccal G forms from differentiated cells of the S or R types.

If it be conceded on the fore-going evidence that the G forms do not represent a unique contaminant or a foreign species symbiotically associated with the parent cultures, but are in fact produced as true variants of a single species, it becomes necessary to enquire whether on the one hand they are accidental pathological variations, arising under the influence of various non-specific harmful agencies, or, on the other hand, physiological forms which may conceivably play an important part in the natural history of the species. As Hadley recognised, the former view is one likely to attract many orthodox workers, who he thought might assume that the G forms were stunted variants which merely required a return to favourable environmental conditions in order to revert to the original type. The writer has observed the appearance of forms indistinguishable from the G type in cultures of B. typhosus subjected to the action of such a frankly deleterious agent as izal, but there is a considerable amount of evidence to support the hypothesis that the emergence of the G type is an essentially physiological process and that the use of such stereotyped terms as "involution forms" or "pathological forms" is, in this case, unjustified.

In the first place, both in the present work and in Hadley's study the G type was frequently isolated from cultures in normal broth. Although

the number of cases in which success was attained from the use of lithium chloride was relatively greater, Hadley nevertheless concluded that this substance, as also pancreatin, was able to bring about no change which might not be obtained in plain broth media with young cultures.

Yet another circumstance opposed to the interpretation of such forms as pathological variants is the fact that the use of most dissociative reagents is not incompatible with reversible changes in the composition of the culture. While attention has been directed mainly to changes from the more differentiated forms to forms of lower organisation, as from S to SR, R or O types, Hadley nevertheless observed, in the course of serial transfers in lithium chloride broth, successive waves of both S----->R and R----->S changes, and while these in part at least were probably conditioned by selection processes and only less by actual transformation as he believed, they show that the dissociative agent can only be regarded as harmful in a limited sense. Most dissociative influences appear to act, therefore, not by producing a permanent pathological disorganisation of the cell but rather by inducing a physiological degradation of organisation which, according to circumstances, may or may not be permanent. But in this as in other matters

it should be realised that the boundary between the physiological and the pathological is often ill-defined, and that interpretation must often be arbitrary.

Relation of the filter-passing bodies to the visible forms of the G-type cultures.

It is clear that the visible coccoid bodies seen in G type cultures are manifestly non-filterable, and it is accordingly important to determine the relation which exists between these larger forms and the filterable elements with which they are frequently associated. Hadley demonstrated a relation between the grade of candle employed and the time when G colonies first appeared on plates seeded with the corresponding filtrates. He showed that bodies passing the N and W candles were of such a nature that their regeneration into the visible G culture did not occur, as a rule, until the filtrate had been passed over from three to six agar plates, while filtrates derived from V candles generated the G forms somewhat earlier in most cases and often on the first plate. It is apparent and cannot be overstressed, that the form of the organism passing

suitable filters is very frequently not susceptible of immediate visible cultivation on agar plates, but that a period of development or germination is required before visible forms appear. This metamorphosis can be demonstrated, as has been shown, by serial transfers over fresh agar plates, or, alternatively, by allowing the fresh filtrate to age, when the filterable elements develop the property of visible cultivability. It would seem most likely that the G type is represented by coccal forms of a wide variation in size and of characteristically slow growth-rate, and that while the smallest elements may be separated by filtration, they tend in suitable circumstances to produce detectable colonies whose elements are at the upper limit of size.

The possible relation of G type cultures to  
other culture forms reported in the literature.

There can be little doubt that the commoner types of bacterial variation were frequently encountered before the S----->R variation was described as such and its significance established. It is therefore of some interest to search the older literature for references to variants with any resemblance to the G-type. According to Hadley the notion of the existence of a minute stage of culture development has been dawning in the bacteriological world for many years, and quite apart from his own contribution there seem to be numerous observations in the literature which support the existence of bacterial phases physiologically equivalent to the gonidia of the fungi.

The first description of gonidia-like bodies in bacteria appears to be due to the Swiss Perty (1852). These were observed in certain species of Spirillum and Perty depicted the origin of these bodies (blastia) inside the organism and their subsequent liberation and development into the original cell type.

In 1870 Cohn described and pictured the gonidial bodies in Crenothrix polyspora as micro-

and macro-gonidia. Burdon-Sanderson (1874) stated that there occurred in some of his cultures certain "spheroids" which apparently developed into rod forms, and he presented the possibility that bacteria might be formed from more minute or ultramicroscopic bodies. Billroth, also in 1874, claimed that minute coccus bodies might play an important part in bacterial multiplication, and his drawings depicted the generation, multiplication and final liberation of the gonidial bodies.

It is of great significance that Koch (1877), in some of the earliest micro-photographs ever taken of bacteria, showed (plate XV, fig. 4), what he termed "Bacillen mit mehreren seitlichen Sporen" in Bact. termo. Fig. 5 in his plate XVI shows similar rounded lateral or terminal buds, but Koch made no comment on their nature or significance. Hadley in particular drew attention to the striking resemblance between the "seitliche Sporen" and gonidial bodies.

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Note: According to Lohnis, some authors like Hauser (1885) and Pfeffer (1888) were of opinion that the old Bact. termo should be identified with B. proteus. But Mace (1897), E.F. Smith (1905) and Lehmann and Neumann (1912) defined it as Bact. fluorescens, and this latter view Lohnis believed to be correct.

About this time similar observations and appearances were recorded and described by Cienowski (1877) (for Crenothrix, Cladothrix); Geddes and Ewart (1878) and Ewart (1878) (for B. anthracis); Israel (1878) (Actinomyces); Albrecht (1881) (Spirochaeta obermeieri) and Zopf (1879-1885) (in the Trichobacteria). It is of some importance to note that both Klebs (1883) and Babes (1883) were able to convince themselves that granules produced by acid-fast bacteria might in turn reproduce the bacillary form. Later observations are those of Neisser (1888) on B. xerosis; Beijerinck (1888) on the "swarming bodies" of B. radicumicola; Dowdeswell (1889-1890) on the granules of the cholera vibrio; and of MacFadyean (1889) on the "seed forms" of Actinomyces. Zettnow (1891) published photographs showing the budding of possibly regenerative bodies in bacteria and spirilla, but he regarded them as degenerate globules or "Krankhafte Degenerationsformen" and thus of little significance. Similar forms were regarded by Loeffler as "Absterbenserscheinungen" concerned only with degenerative changes.

Cunningham (1897) demonstrated the reproduction of V. cholerae from coccoid forms, a fact also recorded for the Finkler-Prior vibrio and V. metchnikovii. Almquist (1893, 1911) showed that B. typhosus and B. coli when kept at lower temperatures than usual (10-11 C.) produced minute round bodies which

later reproduced the original form. In 1911 he recorded the development of small coccal forms, which he regarded as gonidia, from the rods and filaments of a B. typhosus culture. These in turn became transformed into minute granular bodies ("Kornchen") which were filterable through a Berkefeld candle. In 1904 Almquist had described larger round bodies which budded from the sides or ends of the vegetative rods, and which he termed "conidia". These conidia were able to multiply as such by budding. In 1916 he again pictured the conidia of B. typhosus and B. dysenteriae.

Lohnis (1921) drew attention to a rather inaccessible Russian thesis by Rohtert (1902) on "Degeneration and regeneration of the bacteria". In this contribution bacteria were described as producing relatively resistant granules during periods of "degeneration", and it was claimed that these might regenerate under suitable conditions. Such a process was studied and described in the following species: Staphylococcus pyogenes aureus, Streptococcus pyogenes, B. coli, B. typhosus, B. pneumoniae, B. capsulatus Pfeiffer, B. rubidis, B. arborescens, V. cholerae and V. Milleri. Fedorowitsch (1902) has also been quoted as describing germination of the "granules" produced by B. pyocyaneus, B. typhosus, B. coli, B. septicaemiae murium, B. cholerae gallinarum, B. diphtheriae, and B. tuberculosis.

For these regenerative granules he proposed the term "protospores" since they appeared to represent reproductive organs similar to endospores, though not possessing the high resistance of these latter forms. Chester (1901) described the granular particles which replace tubercle bacilli in old lesions, and regarded them as "resting bodies of the nature of gonidia, which are capable of reproducing the species." These particles are probably identical with the fragments or "Splitter" of the tubercle described by Spengler (1905-1907) and thought by that author to have the nature of spores.

The literature contains numerous descriptions of granular or globoid bodies appearing in relation to, and apparently derived from, various spirochaetes. In 1906 Novy and Knapp claimed to have demonstrated the filterability of such forms in Spirillum obermeieri and related organisms, and from papers by Dutton and Todd (1905, 1907), Leuriaux and Geets (1906), Krienitz (1906), Breinl and Kinghorn (1906), Wolbach (1915), Perrin (1906), Breinl (1907), Leishman (1909), Balfour (1911), Hindle (1911), Fantham (1911), Nicolle and Blanc (1914) and others, it seems more than possible that the metachromatic or "chromatine" granules within the spirochaetes may multiply as such or may eventually become transformed into the typical spirochaetes. As has been pointed out by Hadley among others, such a view

accounts for much that is otherwise obscure in the behaviour of such spirochaetes in the course of infection in the living host. Of special interest in this connection is the work of Leishman (1909), particularly in view of the excellence of his photographic illustrations. After the ingestion by Ornithodoros moubata of blood containing S. duttoni, the latter soon lost their motility in the intestinal sac and, eventually, their characteristic appearance. Morphological changes occurred in them which resulted in the formation and liberation of small chromatin bodies --- rod-shaped, coccoid or curved in form --- which divided in the tick tissues and were proved to be infective. That a similar process takes place in the life history of Treponema pallidum has been argued by Levaditi among others, and according to Levaditi, Schoen and Vaisman (1934), "les deux phases caracteristiques du cycle evolutif du virus syphilitique, la phase aspirochetienne infravisible et la phase treponemique, evoluent de la meme maniere, peu importe si le materiel d'inoculation contient, ou non, au prealable, le Treponema pallidum."

In 1921 appeared the monumental work of Lohnis which, although highly uncritical in conception, nevertheless contains the bulk of information on this subject to the date of its publication. Lohnis believed that all bacteria multiply not only by fission but also by the formation of filterable

gonidia. According to him these bodies, in the form of regenerative particles, or occasionally exospores, are liberated from the parent bacterium by a partial or complete dissolution of the cell wall and may reproduce the original type after a varying interval. D'Herelle (1922) described the occurrence of filterable forms of the Shiga bacillus in the filtrates of lysed cultures of this organism, and similar forms were reported for other species (B. coli, B. typhosus, B. dysenteriae), also under the influence of homologous phage (Hauduroy 1924; d'Herelle and Hauduroy 1925). These forms were described as yielding a faint opalescent growth in broth, or a delicate growth on solid media, and reversion to the original bacillary form, or to a modified cocal type, was also described.

In a study of the biology of B. anthracis Haag (1927) described and pictured gonidial forms and indicated their modes of origin and liberation from the parent cells. Oesterle and Stahl in 1929 recorded similar findings for B. mycoides, in which species they described the production of filterable gonidial forms under the influence of sunlight, sodium chloride, mercuric chloride and chloramine-Heyden. These forms appeared as minute granular coccoid bodies which grew poorly in culture but eventually reverted to the original parent type

by way of intermediate forms.

Cunningham (1931), in a study of the physiology of the soil organism B. saccharobutyricus von Klecki, depicted structures resembling the "seitliche Sporen", and described them as spherical gonidangia (See Figs. 56, 57). Similar bodies, together with free 'gonidia', are shown in Fig. 58, from an organism described by Evans (1932).

Hadley (1933) has discussed the relation of the bacterial variants of Kuhn to the chief phases in microbial dissociation. Kuhn described five variant forms, A, B, C, D and F, and based his classification on the morphology of the cell rather than the colony characters. Kuhn's B forms (Bakterien-Formen) comprised the ordinary rods regarded as the 'normal' forms of most bacterial species. These he regarded as developing from the C forms (Kokken-Formen) which were represented in turn as a group consisting chiefly of coccal or granular bodies, the smallest of which were minute and filterable, and whose colonies were always small and sometimes microscopic.

The field of bacteriology presents several instances of apparent symbiosis in certain of which the real biological nature of the association is obscure. In particular it is frequently difficult to determine whether the symbionts are genetically

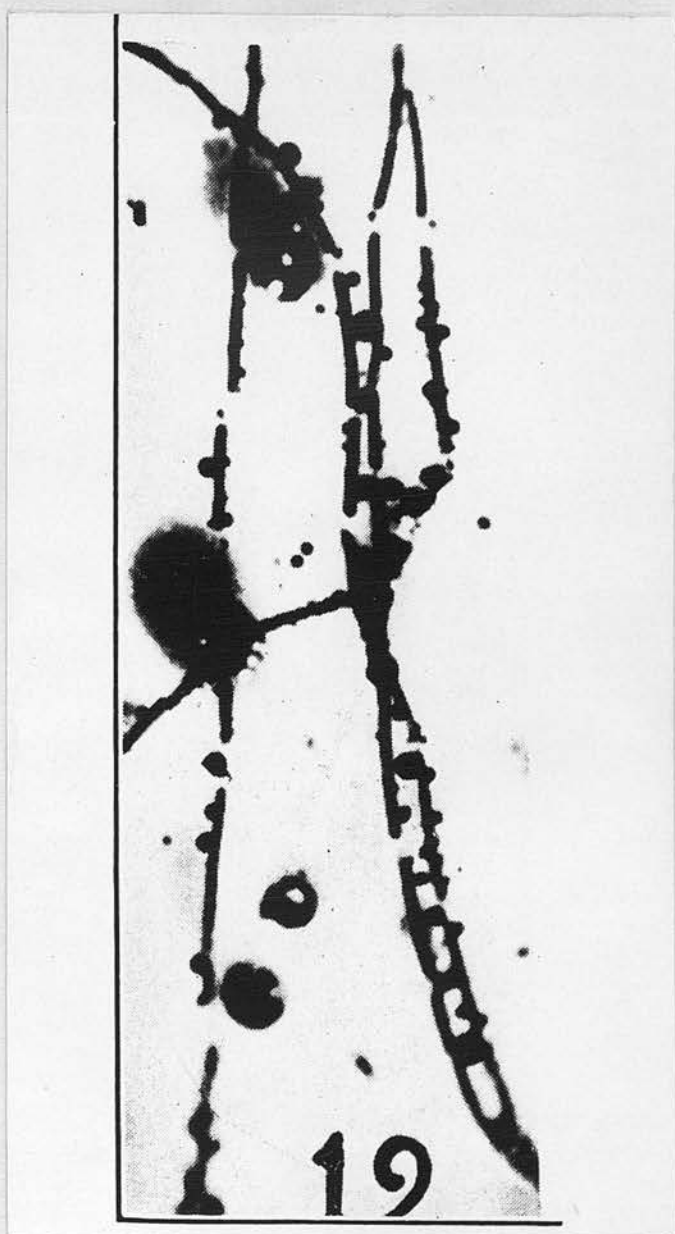


Fig. 58. Gonidia-like bodies  
in the organism described  
by Evans (1932).

related forms or merely exist together in an adaptive relationship of two independent species. An instance of special interest is that of B. actinomycetem comitans (Klinger) which, as a minute Gram-negative and avirulent coccoid organism, is frequently associated with the mycelial filaments of Streptothrix actinomyces in actinomycotic lesions and cultures. Colebrook (1920) observed its occurrence in the majority of the cases he studied, and it has been frequently noted by other workers. Its outstanding features are that it can be cultivated separately from the actinomyces both aerobically and anaerobically and that its biological characters present a striking contrast with those of the streptothrix. While it is easy to separate this organism from the actinomyces by the ordinary methods of isolation, in many cases it has proved difficult to obtain pure growths of the mycelial organism free from the concomitant. Muir and Ritchie (1932) referred to two strains in which it was found impossible to separate the mycelial organism from the concomitant even after repeated plating and sub-culturing from single isolated colonies. The suggestion has been made that in this case the concomitant organism represents a true derivant or variant of the mycelial organism, although the wide divergence in the biological characters of the two types has proved a bar to the acceptance

of this view by most workers, who prefer to regard these organisms as individual species. This possible objection loses most of its force however when we consider the wide range of variability which most micro-organisms are now known to possess. As an example may be cited the G forms described in this contribution and by Hadley and other workers: if encountered separately these would undoubtedly be classified as species totally remote from those of their parent cultures. It is of some significance that the older literature contains several references to the formation of minute intracellular bodies within the actinomyces mycelium, which escape and reproduce for a time (Bostroem, Miller; quoted by Novak and Henrici 1933).

Although their precise significance in this connection is not at present altogether clear, mention may be made of the remarkable phenomenon of the production of amorphous extra-bacterial substances in bacterial cultures, originally described, in the case of a fluorescent milk bacillus, by Oerskov (1931, 1937) and studied in detail by Dienes (1935, 1937). According to the latter author the extra-bacterial substance was produced in saccharose-containing media and was actually composed of myriads of granules which could be passed in large numbers through Berkefeld filters. Dienes demonstrated

the actual process of the liberation of granules from the bacteria. In one strain there developed secondary colonies consisting mainly of transparent material, and when this was transferred to other plates it gave an abundant growth of granular colonies without any bacterial growth. Oerskov at first believed that the non-stainable and multiplying granules existed in symbiosis with the organism, but Dienes disagreed and found that the granules were actual derivants of the bacteria. This phenomenon may or may not be related to certain observations made by Broadhurst (1933) on the occurrence of amorphous phases of bacteria. In her experiments however no filtration experiments were carried out.

It is thus evident that the literature contains numerous descriptions of processes which may justifiably be interpreted as the generation of living fragments from bacteria: while frequently stable in so far as they may multiply as such for considerable periods, these fragments are in some cases at least, potentially regenerative. Fig.<sup>59</sup> shows the various morphological aspects of budding and branching as depicted by Meirowsky (1914). Ruzicka (1907, quoted by Lohnis 1921) was of opinion that such regeneration of normal bacteria from living fragments had not received adequate attention, despite

	Seiten- und endständige Knospen	Dolden- bildung	Freie Knospen und Jugend- formen	Teilung der Knospen und Längsspaltg.	Seiten- sprossung
Tubercul. hum. Reinkultur					
Tubercul. bov. Reinkultur					
Leprabazillen aus einem Leprom nach Behandlung mit 10% Antiformin					
Paratyphus B. Reinkultur					
Gärtner'sche Enteritisbazillen Reinkultur					
Spirochaeta gallinar.					
Mundspirochäten (Stomatitis mercur.)					
Spirill. rubr.					
Spirochaeta pallida Reinkultur					

Fig. 59. Morphological aspects of bacterial budding and branching (Meirowsky 1914).

the important analogies displayed in the life of other primitive plants and animals. There can be little doubt that a wide variety of slightly detrimental influences ( as for example a suitable concentration of lithium chloride), which reduce the vitality of the vegetative cells, result in the appearance of atypical forms: a common though superficial interpretation is to regard all such forms as pathological and produced say by the simple physical effect of plasmoptysis. Another frequent criticism dismisses such variants as contaminating species. Since most of the forms described as gonidial are micrococcal in morphology and not infrequently manifest profound alterations in their staining reactions and other physiological properties as compared with the parent culture, such criticism is perhaps more easy to maintain than any other, although actually in many cases the writer believes it to be facile and superficial. No one would wish to deny the supreme importance of scrupulous technique in the study of variation, and excellent reviews of the subject have been given by Frobisher (1933) and Holman and Carson (1935). But criticism of this kind which is constantly based on the contamination hypothesis seems to the present writer to fail because it ignores the wide potentialities for morphological and physiological variation which all organisms, including micro-

organisms, undoubtedly possess. It was found in certain experiments for example that exposure of B. typhosus to a suitable concentration of izaral led to the appearance of Gram-positive micrococcal forms and gave a microscopic picture which any bacteriologist unaware of the circumstances would have described without hesitation as a mixed culture; yet in these experiments, quite apart from the question of interpreting physiological nature, there could be no doubt as to the real production of a morphological variant.

Note on nomenclature.

While the phenomenon of gonidia-formation has been referred to as such in many of the more authentic cases in the literature, descriptions of the same or allied phenomena are frequent, especially in the older literature, in which the alleged gonidia are variously referred to as "blastia", regenerative bodies, micro-gonidia, swarm-spored, microzymas, microsomes, microblasts, sporoids, infective granules, and conidia. It should be pointed out that the use of the expression "gonidial spores" and the occasional synonymous use of the terms "gonidia" and "spores" do actually indicate a certain physiological similarity in the processes of gonidia- and spore-formation. This similarity is best evidenced by

their properties of regeneration and relative heat-resistance. The latter property has been frequently recorded, and as an example may be mentioned the statement of Spratt (1912) that the round regenerative bodies of B. radiculicola remained alive even in boiling water. Etymologically there seems little reason to make a wide distinction between the terms "gonidium" (derived from γονος, offspring) and "spore" (from σπορα, seed), but the technical difference which undoubtedly exists is best indicated by the use of the distinct term "endospore" for the reproductive stage of the acknowledged sporing bacteria. Confusion of a rather different kind has been due to the synonymous use of conidium (from κονια, dust), but it seems preferable, on the suggestion of Lohnis, to omit this term completely as far as bacteriology is concerned.

#### General considerations.

The general hypothesis, that in certain circumstances many bacterial species may propagate by budding or gonidia-formation as well as by simple fission has obvious implications in regard to what has been called the time-honoured conception of the Schizomycetes. But its systematic importance is rather to lessen the physiological differences between the Schizomycetes and Actinomycetes, and to

indicate a functional continuity the basis of which lies probably in the fact, pointed out by Darwin (1868, quoting Huxley), that fission and budding are "essentially alike", that budding may be regarded as a special case of fission, or that "fission is little more than a peculiar mode of budding."

Relation of G type cultures to bacteriolysis.

At a time preceding or accompanying the first appearance of the G forms, Hadley frequently recorded the disappearance of the S type elements in the culture, and from such observations he was led to discuss the nature of bacterial autolysis.

In its orthodox use, the term "bacteriolysis" usually connotes a disintegrative change leading to death of the bacterial cells, and as such has been studied in old cultures and in organisms such as V. cholerae and B. typhosus under the influence of immune serum. Notwithstanding this common interpretation, various investigators have from time to time produced evidence to indicate that autolysis brought about by such agencies does not inevitably result in the death of all the cells affected, but that in some cases there may result a transformation

of the cultural type. On these grounds it seems reasonable to conclude, as Hadley suggested, that the phenomenon of bacteriolysis may represent two quite distinct processes, one an indication of real degeneration and the other representing a stage in the transition of the cell into smaller viable units.

Such views are of particular interest in the special case of bacteriophagy, since in this case the normal cells may entirely disappear from view as if their destruction were complete. It is equally likely on the other hand that the living germ-plasm of the culture is never entirely destroyed in this way, and it seems indeed as though the action of the bacteriophage is to transform rather than annihilate. Reference has already been made to the striking experiments of Kendall and Walker (1933), which obviously give considerable support to the view that the products of phage-induced lysis include minute filterable bodies which in a suitable environment are able to regenerate the original parent type. Hadley suggested in addition that this reversion might proceed by way of the G type.

These considerations are of special interest in relation to Hadley's homogamic theory of the nature of bacteriophage (Hadley 1928). According to this view the bacteriophage was postulated as a filterable

cyclostage in the life history of the culture concerned or of a closely related species. The question then arose as to the relation between the filterable elements preceding or associated with the G type cultures and the equally filterable bacteriophage. In 1928 Hadley and Klimek claimed that the bacteriophage could be produced artificially in 'normal' cultures by inducing dissociative changes (see also Hadley and Buonaventura 1931), and after the discovery of G type variation the general problem became defined as "the possible relation existing between the bacteriophagic corpuscles and the filterable bacterial forms which co-exist with the chief elements of the G-type culture."

In certain experiments, the details of which were unpublished, Hadley and his associates attempted to study the spontaneous appearance of bacteriophage for the Shiga bacillus by repeated growth of the stock S type culture in its own filtrates, and obtained a positive result once after the twentieth serial filtration and twice after the fourteenth. It was further found that when pancreatin broth was used as a medium and the same method employed, the bacteriophage was spontaneously generated with remarkable regularity at about the eighth serial filtration. It will be recalled that Hadley found, in attempts to induce the appearance of G forms by serial transfer through lithium chloride

broth, that these forms were generated most frequently between the sixth and twelfth serial tubes, and Hadley accordingly attached considerable significance to this relation by which, under constant conditions of experiment, was determined the time of appearance not only of the elements of the G type cultures but also of the corpuscles of the bacteriophage. He suggested that the bacteriophage corpuscles might in some way be associated with the filterable elements of the G type and in any case thought we must regard both the lytic units and the G elements as potentially present in any 'normal' culture and recognise the fact that the same influences (enforcement of dissociation) might serve to liberate both. Along these lines, Hadley postulated the following questions.....What is the relation of the bacteriophage to the filterable, but eventually cultivable, organisms that arise in lytic filtrates? What is its relation to the filterable forms of the G-type cultures, obtained without the recognised intervention of bacteriophage acting on the original cultures? What is the relation of the filterable and cultivable bodies obtained from lytic filtrates to the filterable and cultivable elements obtained from cultures undergoing the dissociative reaction? When these questions were answered, he claimed, the nature and origin of bacteriophage would be much less of a mystery.

In view of the possibility that the bacteriophage corpuscle may originate intrinsically in the bacterial cell, it may be of some interest at the present juncture to interpolate a brief summary of the present position of the problem in general.

The available data can be divided into two main classes: (a) those the significance of which is indubitable, and which are accepted and interpreted similarly by different schools of opinion; and (b) data which, in the absence of fuller details, are open to interpretation in two or more ways. In the first class belong facts relating to the particulate nature of bacteriophage, and to its size, organisation and antigenicity. The particulate nature of the agent may now be regarded as completely confirmed, especially in view of such critical experiments as those of Feemster and Wells (1933; see also Levaditi, Paic, Voet and Krassnoff 1936; and Gratia 1934). As regards size, Elford and Andrewes (1932), applying the methods of filtration through graded collodion membranes, found that while the particles of any single pure phage appeared to be of uniform size independent of the bacterial strain used in their production, individual phages showed relatively great differences in size. Thus the phages lysing dysentery bacilli range in size from 8-12m $\mu$ , (the size of the virus of four-and-mouth disease), to 50-75m $\mu$ , the intervening

range being represented by an almost continuous series of particle diameters. This order of size connotes another property which one may designate as organisation, and indicates the possibilities of actual optical demonstration of the larger corpuscles. This last has actually been accomplished by Burnet (1933 b), using microphotography by monochromatic ultraviolet light, to detect the aggregates obtained when dysentery phage is treated with homologous antiphage serum. Lastly, no possible doubt can exist regarding the antigenic capacity of purified phage. That specific antibodies can be readily obtained by the ordinary processes of immunisation affords presumptive evidence of the presence of protein in the bacteriophage unit, and, as Burnet showed (1933), while the specific antigen is found mainly on the surface of the particle, it can also exist in soluble form and may be detected in phage-free ultrafiltrates of lysed cultures.

Thus far the present position indicates almost complete conformity of opinion, but this is strikingly absent in regard to the interpretation of other phases of the activity of bacteriophage, and the cleavage of opinion is more pronounced in any discussion of the precise biological relationship between phages and bacteria. Not the least notable feature of phage is its relatively strict limitations of specificity, by which a dysentery phage may be completely devoid of

action against say a staphylococcus or cholera vibrio. A similar phenomenon is the parallelism which is found to exist between the properties characteristic of a given phage and those characteristic of the corresponding bacterium. As extreme instances of this association may be mentioned the description by Koser (1926) of a bacteriophage acting optimally at 55° C. to 58° C. on a thermophilic organism the optimum growth of which occurred between 45° C. and 52° C., and the report by Elder and Tanner (1927) of a bacteriophage operative at 4° C. or below on an unnamed psychrophilic species which grew well at 4° C. but not at 37° C. Craigie and Brandon (1936 a, b) found that bacteriophage specific for the V form of B. typhosus might be present in cultures of this organism derived from the stools of cases and carriers, and exposure of the V form of the organism to V phage resulted in conversion to the phage-resistant W form, with loss of V agglutinin and capacity to absorb the phage (see also Sertic and Boulgakov 1936 a). Sertic and Boulgakov (1936 b) also described phages specific for flagellate bacterial varieties, and the production, under the influence of such phage, of non-flagellate variants. Such parallelism may obviously be regarded on the one hand as evidence of an adaptive symbiosis between an organism and parasite or alternatively as a hint of the possible derivation of phage from the bacterium itself, since their biological characters possess so much in common.

The mechanism underlying specificity was first studied in adequate detail by Burnet (1927), who found that B. typhosus, B. enteritidis and B. pullorum, which possess in common an O (polysaccharide-containing) antigen but are otherwise dissimilar, were sensitive to a type of bacteriophage which was without action on any strains lacking this antigen, including the R variants of the sensitive S strains. On the other hand (Burnet 1929 a) it was possible to obtain resistant variants which showed no detectable antigenic difference from the sensitive parent strain, and Burnet accordingly concluded that "if phage reactions are primarily determined by the molecular configuration of the antigen they must be more susceptible to slight changes than are the serological reactions." Burnet now concludes that the nature of the bacterial component responsible for the adsorption of phage is closely related to and perhaps identical with the specific polysaccharide-containing antigens.

Supporting evidence of a similar kind is due to the work of Levine and Frisch (1933) on the phage-inhibiting activity of bacterial extracts, by which it is shown that extracts from autolysed bacteria can inhibit the activity of phages which are capable of lysing the strain in question (see also Sertic 1937). The factor concerned in this reaction possesses the same specificity towards phages as is shown by the

bacteria from which it is derived, and it is therefore regarded as the actual surface component of the bacterium which determines adsorption of phage as a preliminary to lytic action. It is of interest that Lancefield (1933) found that susceptibility to a streptococcal bacteriophage was correlated closely (though not absolutely) with the possession of a particular carbohydrate antigen. Pandit, Maitra and Datta Roy (1936) found that extracts of strains of true cholera vibrios inhibited some of the ten types of cholera-phage they used. No definite correlation could be found between phage type resistance and corresponding type inhibition, but multiple type resistance was associated with a diminution in the number of types inhibited. Extracts were classified into three groups according to their phage type inhibitions. These were found to be generally similar to the groups obtained by Linton according to the respective polysaccharides of these strains determined by chemical analysis. Bruce White (1936) thought it probable that one cholera phage (A) is specifically bound by the smooth polysaccharide of V. cholerae, and on this makes its primary attack, while certain other phages (notably C, E, G and H) are fixed by and possibly make their first attack on, the lipid constituents of the vibrio. Scholtens (1936, 1937) described two typhoid phages, one of which lysed Bact. typhosum alone, while the other lysed this

organism and also Bact. enteritidis. The tentative conclusion was drawn that the first probably attacks the Vi antigen and the second the somatic antigen IX of the Kauffmann-White classification.

While such a strikingly specific mechanism exists to explain the adsorption of phage by sensitive bacteria, it is important that there is apparently no demonstrable community of antigenic structure between a bacteriophage and its homologous culture. As early as 1925 Wollman and Brutsaert attempted to relate the bacteriophage antigen to the antigenic constituents of the susceptible bacteria. They found however that antibacterial sera did not possess antilytic powers, while these were manifested by the antiphage sera, and concluded that the bacteriophage must be regarded as a new antigenic entity, not found in normal bacteria. As Hadley has pointed out, however, (1928), this negative evidence does not exclude any given interpretation of the biological relationship between a given bacteriophage and its appropriate culture.

Still another biological phenomenon is of undoubted significance for the eventual solution of the problem of bacteriophage, although for the present it serves merely as a further controversial topic. This refers to the property of lysogenicity, displayed by many bacterial strains, in virtue of

which they liberate phage during their growth. In a series of 130 stock laboratory strains of various Salmonella types Burnet (1932) found that at least 93 regularly produced phage capable of lysing one or more of three "indicator" strains. The following quotation (Burnet 1934) serves to show the importance of this phenomenon and is of special interest as coming from a worker whose viewpoint is essentially similar to that of d'Herelle and whose interpretation of the biological status of the bacteriophage is to regard them as "a class of diverse micro-organisms whose common feature is ability to parasitise or live in symbiosis with bacteria."

"Lysogenic strains.....produce phage consistently despite all attempts to free them from this property by such means as repeated re-isolation or growth in appropriate anti-phage serum. Even when variants of the strain are tested they still retain the lysogenic attribute. One lysogenic strain of B. enteritidis gave rise to a whole series of variants, smooth and rough, motile and non-motile, mucoid and pellicle-forming, but all gave the same phage when grown in broth culture, only differing in the average amount produced. It is obvious that in such cultures as this every cell must contain some rudiment of the phage which is liberated in growing cultures. If the organismal nature of bacteriophages is true for those liberated from lysogenic cultures as well as for the

rest, and some unpublished filtration experiments indicate that the phage released from the enteritidis strain mentioned above has a particle size commensurate with that of other phages, one is almost forced to postulate that each bacterium carries in intimate symbiosis one or more phage particles which multiply by binary fission pari passu with the bacterium. Further, the multiplication of phage and bacterium must be so co-ordinated that each daughter individual regularly receives the phage. Where the bacterial component of the symbiosis is a spore-former it can be shown that the lysogenic attribute is retained by the spore and is resistant to any degree of heat which still leaves the spore viable (den Dooren de Jongh 1931; Cowles 1931). The increased resistance to heat of the phage held within the spore can probably be correlated with the relative dehydration of the spore.....On the whole it is not surprising that most of those who have studied such lysogenic bacteria are in favour of the view that the liberated phage is not an autonomous micro-organism but some unit of the bacterial "chromatin", a vagrant gene freed from normal restraint." (For a discussion of induced lysogenicity and "mutation" of bacteriophage within lysogenic bacteria see Burnet and Lusk 1936).

Nevertheless Burnet himself, on the basis of a previous investigation (1932) decided that such a conclusion was inadmissible. Of twenty-four lysogenic

strains of B. enteritidis Gaertner, fourteen gave rise to B phage, seven to D phage, two gave both A and B phages and one A and D. The same random type of distribution was obtained with paratyphosus A and B and aertrycke strains. "In other words with these species the nature of the phage produced was not determined by the nature of the organism from which it was obtained." On the other hand Burnet found in the same investigation that another group of Salmonella organisms, the suipestifer type and its relatives, showed a common lysogenicity and all the available strains of paratyphosus C, including examples isolated in Russia, South America and the East Indies, produced similar amounts of an identical phage. Assuming the symbiosis theory to be correct, Burnet acknowledged that this must represent an extreme degree of intimacy of association.

Another aspect of the subject, closely related to lysogenicity, is the alleged production of bacteriophage from normal bacteria. Burnet agrees, as must the most confirmed upholder of the parasitic theory, that "if a method were available which would regularly induce the appearance of phage from all cultures of those bacterial species which are sensitive to known phages, it would provide adequate evidence to discredit the micro-organismal theory of bacteriophage." As is often the case in similar

problems, the question is not easily amenable to experimental proof or disproof, mainly because of two objections, both of which were originally raised by d'Herelle. These are (a) the difficulty of ensuring that a given organism is devoid of bacteriophage at the commencement of the experiment, and (b) the possibility of contamination of the test culture by a laboratory or extrinsic bacteriophage during the course of the experiment. Mention has already been made above of certain experiments by Hadley which that author regarded as affording some support to the suggestion that normal bacteria are capable of elaborating bacteriophage, but even more remarkable is the recent description by LeMar and Myers (1935) of a method, which, it is claimed, leads to the artificial production of a specific lytic agent from a wide variety of different organisms. Cultures of B. typhosus, S. paratyphi, S. Schottmulleri, S. enteritidis, E. dysenteriae, E. paradysenteriae, E. coli, Staphylococcus aureus, Staphylococcus albus, several strains of haemolytic streptococci, Micrococcus flavus, Pseudomonas aeruginosa, Aerobacter aerogenes, Haemophilus influenzae, Proteus vulgaris, and pneumococcus, were grown in peptone broth, blood broth and brain broth, according to individual nutritive requirements, for 48 hours at 37° C. They were then killed by autoclaving for twenty minutes at 15 lbs. pressure and re-incubated at 37° C. for

48 hours. Ten c.c. of 3 per cent. hydrogen peroxide per 100 c.c. of culture were added, and the cultures again incubated for 48 hours at 37°C. Ether extracts of the cultures were then made and found to contain lytic agents specific for the homologous organism. These were transferred serially for seventeen culture generations without diminution of lytic power, and the formation of plaques on solid medium was also described. All were destroyed by exposure to 75°C. for thirty minutes and could not be rejuvenated by serial transfer. In no case could hydrogen peroxide be detected in the extracts. Additional experiments showed that oxidation of living cultures yielded little or no lytic agent, and filtrates made from cultures which had been autoclaved and permitted to incubate the second time without oxidation were not lytic.

It is obvious that results so completely unusual are at variance with orthodox opinion and belief. Yet while attempts to confirm these findings are urgently called for and will no doubt be forthcoming in due course, it seems possible that the phenomenon reported by LeMar and Myers has a definite foundation in fact. From the descriptions given by these authors it will be noted that, while a living and specific lytic agent was generated presumably from the bodies of autoclaved bacteria, after subsequent propagation the lytic agent was destroyed by an exposure to a temperature of 75°C. for thirty minutes.

In view of Hadley's claim that the G type organism and the bacteriophage are closely related and represent cyclostages in the development of 'normal' bacteria, it is of especial interest that the phenomenon described by LeMar and Myers exactly parallels the isolation of G type organisms from autoclaved cultures of B. paratyphosus B described above. In the latter case the incubation of autoclaved cultures was followed irregularly by the appearance of typical G type elements identical in all respects, morphological, biochemical and serological, with the G form originally isolated as a spontaneous variant. In this case also, while as far as could be ascertained the coccal elements appeared to generate de novo from the autoclaved bacilli, they proved only moderately heat-resistant after subsequent propagation. While the isolation of G forms from autoclaved cultures of B. paratyphosus B was definite and striking, unaccountable factors apparently made it irregular and difficult to reproduce. Thus while repeated experiments gave several positive results within the space of a few weeks, similar experiments carried out some months later proved completely negative. The apparent generation of bacteriophage in the work of LeMar and Myers seems a constant effect, and it may prove that their technique (growth in suitable medium for 48 hours, autoclaving at 15 lbs.

for 20 minutes, secondary incubation for 48 hours, and oxidation), affords the necessary conditions for an easily reproducible positive result. In this connection mention must also be made of the recent report by Kendall (1936) that specific phage may be elicited by the use of autoclaved sewage as a starter.

If results of this kind are substantiated by future work in the next few years they must undoubtedly produce a complete revolution in the fundamentals of theoretical biology, as well as indicating certain obvious practical applications. As regards bacteriophage itself they will undoubtedly lead to a return to favour of the intrinsic theories such as those of Bail and the Wollmans. The former regarded the phage particles as "Splitter", fragments of the essential hereditary determinants ('Erbmasse') of the bacteria, capable of some degree of independent existence. Bail imagined them to be liberated, to become selectively adsorbed to bacteria possessing similar units, and so to affect these units that their normal balance of metabolism was displaced so as to produce a disruptive action on the organism. Wollman and Wollman (1932) summarised the findings which in their view supported the hypothesis of hereditary factors as the only satisfactory explanation of the principal manifestations of bacteriophage action, and Wollman (1934, see also 1935, 1937) defined his interpretation of the general

position as follows: "Dans l'hypothèse des 'facteurs héréditaires' que nous avons développée à diverses reprises, et à la lumière des données que nous venons de résumer, la bactériophagie apparaît comme un phénomène à deux temps. Le premier est celui où interviennent les Bactériophages; éléments d'origine bactérienne porteurs d'un caractère héréditaire, ils impriment aux cellules qu'ils atteignent une variation se traduisant, entre autres caractères, par une activation ou une surproduction des autolysines normales. Ce sont celles-ci qui déterminent le second temps, celui de la lyse ou plutôt de l'autolyse proprement dite." In later work (1936 a, b), Wollman and Wollman confirmed the findings of den Dooren de Jong on the persistence of the lysogenic function in B. megatherium cultures derived from spores heated at 90° C, although they interpreted the phenomenon as a regeneration of bacteriophage rather than simple persistence or survival. Wollman and Wollman (1935) described the liberation of bacteriophage through the action of lysozyme on the cells of a lysogenic culture and further claimed to have demonstrated a simple numerical relation between the lysogenic bacteria and the particles of phage. Gratia (1936 a) found himself unable to verify either of these findings, and pointed out what he considered to be serious errors in the Wollmans' technique. Although the subject was further taken up

at considerable length by both Gratia (1936 b, c) and Wollman (1936), the former consistently failed to obtain liberation of intracellular phage by dissolution of lysogenic B. megatherium with lysozyme, in spite of numerous attempts.

The possibility has already been mentioned in the work of Kendall and Walker (1933) that the bacterio-phage corpuscle, or an element associated with it in bacteriophage filtrates, may revert to the normal bacillary form from which it was derived, and attention may be drawn to an identical result obtained by an entirely different method. (Souknev and Volferz 1933). These authors dried active lytic filtrates of Flexner phage on plates and then inoculated these with Sarcina alba, S. rosea, S. aurantiaca, and Staphylococcus aureus. Control plates were treated with phage alone. After six or seven days incubation, small colonies appeared in the neighbourhood of the "cultures-nourrices". These showed granular cocco-bacilli and rods. The characters of these colonies and organisms showed no morphological or serological resemblance to those of the Flexner organism, but after several subcultures strains were obtained the fermentative characters of which conformed more or less to those of B. dysenteriae Flexner, and which were agglutinated by a specific anti-Flexner serum. According to these authors

the phenomenon of bacteriophagy consists in the transformation of the susceptible organism into invisible filterable forms.

Of great significance from both the theoretical and practical aspects is the suggestion that bacterial strains may be susceptible to the action of a characteristic number of bacteriophage types. Morison (1932, 1935) classified bacteriophages as races (or genera) and types (or species), and has employed the phenomenon of induction of resistance to the analysis of bacteriophage mixtures. "If we use a strain of cholera vibrio lysable by all our types and grow it in tubes of broth each containing one of the types of phage, we get strains of cholera, each of which is resistant to one type of phage and is lysable by all the other types. Again, if we grow a susceptible cholera vibrio with all but one of these types, it becomes resistant to all but one of the phages. Using a set of such resistant strains of cholera, we have a rapid and reliable method of analysing any cholera phage filtrate into its component types." The details of such work are compatible with the parasitic or intrinsic interpretations of the origin of phage and do not of themselves give support to one hypothesis more than the other. But their factual significance is obvious, and will become greater when the major question of the precise relationship between bacterium and lytic

corpuscle is finally solved. Contrary opinions as shown in the literature merely represent the reactions of different minds to a body of evidence which can only be regarded as incomplete in certain essential details. Thus while the Wollmans in 1932 looked on the parasitic theory of bacteriophage as untenable, Burnet (1934) thought the theories of intrinsic origin had become progressively more difficult to maintain.

Of great potential importance is the recent description by Northrop (1936) of the concentration and partial purification of a protein with some of the attributes of phage. Schlesinger (1936) was able to produce staining of bacteriophage in the Feulgen reaction, and also likened the bacteriophage substance to chromatin on account of its high affinity for basic dyes. Krueger and Mundell (1936) discussed the possible protein structure of phage, drew attention to the likelihood that heat-inactivation of phage is closely connected with protein denaturation, and also described the re-activation of thermally-inactivated phage. Williams (1936) advanced the interesting theory, with a brief consideration of the supporting evidence, that phage may represent a suspension of extremely small crystals of one or more of the compounds in the homologous bacteria. Bacteriophagy

might thus consist of the seeding of these (amorphous) compounds by phage particles and their subsequent crystallisation.

Eastwood (see Repts. on Publ. Health and Medical Subjects, no. 18), discussed transmissible autolysis in relation to bacterial variation, and put forward the hypothesis of non-viable mutants as the explanation for the phenomena associated with bacteriophage (see also Rutschko 1936). The recent review of Krueger (1936) also tended to favour the intrinsic theory of the origin of phage.

Relation of G forms to methods of recognising  
sterility of cultures.

The unique biological characters of the G type variant have important and obvious bearings on the question of the tests for sterility of culture media. While this aspect of the subject is possibly at present mainly of academic interest, with little significance for every-day bacteriological technique, yet the fundamental biological implications are of first-rate importance. According to Hadley the demonstration of the actual sterility of a substance or medium or tissue is a matter demanding far more careful attention and a vastly more perfect technique than has been accorded this problem in the past, and failure of apparent growth in liquid or solid media may indicate nothing so far as actual sterility is concerned, except perhaps that the particular form of growth that one has been accustomed to search for is absent.

Of great interest in this connection is the observation made by Barnard (1935) that refined microscopical examination of the deposits which sometimes occur in medium containing horse- or rabbit-serum reveals the presence of minute bodies which, in size and constancy of size (about 0.15 to 0.20  $\mu$ ), in shape and in constancy and regularity of shape, and also in general optical properties, are in no way

different from acknowledged pathogenic viruses. The bodies increased in numbers, and photographs showed them in process of fission. These ultra-microscopic saprophytes were cultivated to the fourth subculture.

The possible relation of filterable forms of bacteria to the etiology of communicable diseases.

It is evident that the phenomenon of G type variation represents a biological principle of widespread if not general significance in bacteriology, and it accordingly becomes of some interest to determine what role these variants may play in the course of active disease. It must be clear to the most conservative that the processes of nature are inexpressibly more complex than is ever apparent, and in no field is this more true than that of bacterial infection. But progress has been slow beyond the stage of the relatively obvious, and even now our notions regarding these matters have advanced but little, only an imperceptible distance beyond the stage first depicted by Koch when he wrote in his classic treatise on wound-infection (Untersuchungen uber die Aetiologie der Wundinfektionskrankheit, Leipzig, 1878), .."A special form of germ is associated with every disease,

and this form remains unchanged irrespective of the number of times the infection may be transferred from one animal to another." But progress to some extent has been more marked in the case of certain diseases than of others, and this is perhaps most true of spirochaetal infections. Thus Mellon (1920) recalled that Noguchi found small coccoid bodies in his cultures of Treponema pallidum, and that Warthin observed similar elements in tissues unquestionably syphilitic but devoid of spirochaetes. Among other authors Zlatogoroff (1924) may be mentioned for his support of the thesis that the phenomena of variation in bacterial species must underlie some of the most important problems in bacteriology and pathology. Apart from any more advanced considerations, it is becoming increasingly certain that the reaction of the host to most infections is such as to produce a change in the bacterial environment which in turn induces the formation of variants. As a further suggestion must be mentioned the possibility of the existence of bacterial species whose commonly recognised or 'normal' forms are harmless but whose unrecognised filterable forms are pathogenic. Although at the present time, all G culture types described have been relatively lacking in toxicity or virulence as compared with the S form, the alternative possibility must also be entertained.

Of particular interest in this connection, at least potentially, is the question of the relation of filterable gonidial elements to the study of the filterable viruses. While most workers undoubtedly envisage the filterable viruses as constituting an independent biological order, analogous with but distinct from the true bacteria, it seems that this position may be short of the truth in some cases at least. In 1925 Nicolle made the tentative suggestion that the filterable viruses might represent the invisible stages of microscopically visible bacteria, into which these viruses might under certain conditions become re-transformed. While such a view is largely speculative, its possible truth is suggested at least by certain natural associations to which reference may be made. It seems unlikely that such a view could be applied to even the majority of the filterable viruses, but it is possible that certain cases may find an explanation on some such basis. Beyond indicating the possibility, Hadley believed that his observations could throw little actual light on this problem; "the results that we and our associates have obtained reveal merely the fact that several species of common pathogenic bacteria are able to enter a stage of development in which they are easily and invariably filterable, presumably ultra-microscopic and, at least in part, and for a limited time, non-cultivable (visibly) in or on common

laboratory culture media; moreover, that success in transforming the virus stage into the common visible form demands a special technique rather different from that commonly applied." According to Friedberger (1927) the typhoid bacillus from post-mortem material when passed in series through guinea-pigs assumed the invisible form incapable of culture in ordinary media. The evidence for the existence of this virus was that guinea-pigs which had been inoculated with it developed pyrexia and thereafter proved resistant to inoculation with virulent typhoid bacilli. Furthermore, the intravenous injection into rabbits of a suspension of the organs of such guinea-pigs taken during the pyrexial period led to the development of agglutinins for B. typhosus. It is of interest that Fejgin (1925 a, b) claimed to have produced a filterable stage of the typhoid bacillus, capable of producing an experimental disease in guinea-pigs, by the action of a weak bacteriophage on a normal typhosus culture. Comparable results were also obtained by Hadley and Carapetian (1933) in a study of the pathogenic effect of G type cultures of B. typhosus, also in the guinea-pig.

Of somewhat related interest are the acknowledged facts of variation in the psittacosis virus. It will be remembered that Levinthal (1930) was aware that the virus bodies in psittacosis varied somewhat in size and that he described them on that account as

Microbacterium multiforme psittacosis. In the same year Coles described intra- and extra-cellular bodies --- the smallest ranging from 0.24 to 0.3 $\mu$  in size --- in the spleen of infected mice. Bedson and Bland (1932) then published a morphological study of the psittacosis virus in which they described an intra-cellular developmental cycle of which the elementary body was looked on as the last phase. The existence of such a cycle was confirmed later in a study of the growth of psittacosis virus in cultures of chick embryo tissue (Bland and Canti 1935). It was also found by Burnet and Rountree (1935) that the virus could be readily propagated in the ectodermal epithelial cells of the developing egg, and impression preparations from membrane lesions again showed the developmental changes in the appearance of the virus described by Bedson and Bland. Bedson and Bland discussed the real interpretation of this phenomenon in a second paper (1934) and concluded that the psittacosis virus should be looked on as a micro-organism with bacterial affinities which in the early stages of multiplication produced larger (and less virulent) forms. (see also Bedson 1937).

An intriguing feature of certain infections is the fact that the condition is either caused by or associated with the presence of two apparently unrelated organisms. Thus "hog influenza" in swine is produced by the joint action of a virus and a

species of visible bacterium (see Report of the Medical Research Council 1933-1934, p. 22), and the same type of association may be operative in acute rheumatism, where streptococci of different kinds have long been incriminated although a filterable virus may also prove to be of etiological significance. (see Schlesinger, Signy and Amies 1935).

Natural associations of this kind invariably lead up to the question of the biological relationship of the organisms involved, and in no case has this been more intensively studied than in the Rickettsia-Proteus problem of typhus. Weil and Felix (1921) first propounded the hypothesis that the Rickettsia might become transformed into Proteus X (see also Felix 1937). This has been supported only by a small minority of workers, although Hadley (1927) regarded the typhus fever Rickettsiae as possibly a dissociated stage of the Proteus organism; and the view almost generally held is that the relation between the two organisms is entirely fortuitous. Fejgin (1927) stated that Proteus X might become transformed into typhus virus under the action of bacteriophage, but this claim has not been confirmed by others. Felix (1933) looked on the infective agent as excreted from the body in the form of Proteus X. Since in this stage Proteus X strains are quite able to persist in nature and in human or animal carriers, he regarded the occasional isolation of X strains

from sources unconnected with typhus infections as insufficient evidence to exclude a specific relation between typhus viruses and these organisms, and looked on the hypothesis which postulates a genetic relationship between Rickettsia and Proteus X as much more likely than the pure chance theory.

In previous papers (Felix and Rhodes 1931; Felix 1933) the thesis had been put forward that antigenically the viruses of various typhus-like diseases correspond to various types of Proteus, and according to Felix (1935) cross-protection had been obtained only with those viruses which possessed the O antigen of type X-19. These included classical typhus, Brill's disease and "fièvre nautique", while the viruses of Rocky Mountain spotted fever and "fièvre boutonneuse" yielded a negative cross-immunity test and did not possess this main antigen. Felix also demonstrated that the results of the cross-immunity tests between the different viruses of the typhus group could be predicted by the agglutination reactions obtained by the various types of B. proteus X. The combination of cross-immunity experiments in the guinea-pig with tests for agglutinin-production in the rabbit was recommended for the analysis of the antigenic structure of different typhus viruses.

Castaneda and Zia (1933) studied the absorption of anti-typhus and anti-Proteus sera with Rickettsia

bodies and Proteus organisms, and found (a) the absorption of typhus sera (human or antityphus horse serum) with Proteus X-19 removed only the Proteus agglutinins leaving the Rickettsia agglutinins intact; (b) the absorption of typhus sera with Mexican Rickettsiae removed the agglutinins for both the Rickettsia and Proteus X-19; (c) while normal or formalinised Rickettsiae were not agglutinated by anti-Proteus serum, these organisms when formalinised and heated at 75 C. became agglutinable by such serum; and (d) the absorption of anti-Proteus serum with Mexican Rickettsiae removed agglutinins for formalinised and heated Rickettsiae but did not affect those for Proteus X-19. They drew the conclusion that there exists a common antigenic factor in Rickettsiae and Proteus X-19 which explains the Weil-Felix reaction. In a further study of this common antigenic factor (Castaneda 1934) a specific soluble substance was isolated from Mexican typhus Rickettsiae which gave, with Proteus X-19 antiserum and typhus human serum, the same precipitation reactions as the polysaccharides extracted from B. proteus OX-19. The specific soluble substance extracted from Rickettsia and Proteus OX-19 Castaneda regarded as likely to be of a polysaccharide nature owing to the strong Molisch reaction obtained with such extracts, the heat stability and the negative biuret reactions. It nevertheless contained 7 per cent. of nitrogen. The

general conclusion from this work appears to be therefore that the factor responsible for the Weil-Felix reaction is a soluble specific antigenic component common to both B. proteus X-19 and the typhus Rickettsiae, and similar results were obtained by Kemp and Cain (1934). It is of relevant interest that Wilson (1934) after conducting an investigation with negative results on the alleged antigenic relationship between organisms of the brucella group on the one hand and of the pasteurella, pfeifferella and proteus groups on the other, concluded that, provided that due precautions are taken to ascertain the normal level of agglutinins in a given host, and that antigenically smooth strains are used, there is every reason to believe that the occurrence of agglutinins in a titre above the normal range of variation is due to infection ---latent, active or past --- with the specific organism in question, or in a few instances with an organism, usually of the same genus, sharing a similar antigen. He added that this conclusion might have to be modified for the occurrence of agglutinins to B. proteus OX-19 in human typhus sera, on account of the continued doubt as to the exact relationship of this organism to R. prowazeki, but pointed out however that if future work proved the truth of Felix's contention that serological types of Proteus X correspond to serological types of typhus virus, even this

apparent exception would fall within the general rule.

A comparable situation, somewhat analogous to that in the example already cited, is suspected to exist in the case of certain of the viruses affecting the central nervous system. Rosenow as early as 1916 noted the filterability of a streptococcus cultivable from poliomyelitic material, and in 1922 he recorded the isolation of a similar organism from the tissues in encephalitis. The latest position of this work is summarised in a recent paper (Rosenow 1935). Evans (1932) described a streptococcus, a filterable form and an aerobic spore-bearing rod as phases of an organism cultivated from cases of epidemic encephalitis and occurring in samples of herpetic and encephalitic viruses. It is of interest to note that Hadley in 1929 succeeded in isolating a greening streptococcus from glycerolated samples of the Levaditi herpes-encephalitis viruses and also from Berkefeld N filtrates of the latter virus (see Hadley, Delves and Klimek 1931, p. 150). Regardless of the possible relation of the greening streptococcus to herpes and encephalitis, he concluded that "a certain stage in the cyclogeny of a greening streptococcus is commonly present in rabbit and guinea-pig brain viruses, and that in certain cases the organism is present in a filterable form."

Duval and Luzenberg (1932-1933) reported results which they believed gave further evidence that the Tunnickliff coccus of measles in vivo (in man, guinea-pig, rabbit, monkey) is extremely minute (virus form) and readily filterable through the Berkefeld N filter, while in adaptation to an invitro environment it becomes the familiar non-filterable coccal form. They believed this interchangeable metamorphosis to be determined by natural environmental factors (in living tissue) on the one hand and by artificial growth conditions on the other. Similarly McKinney (1934) found that a precipitate containing viable forms occasionally developed in Seitz-Werke filtrates of streptococcal cultures, and this occurred when atypical cell division and granule formation were clearly evident in the culture before filtration. The viable forms eventually assumed a visible form, in most cases becoming transformed into diplococci similar to the organism of the corresponding parent culture before filtration.

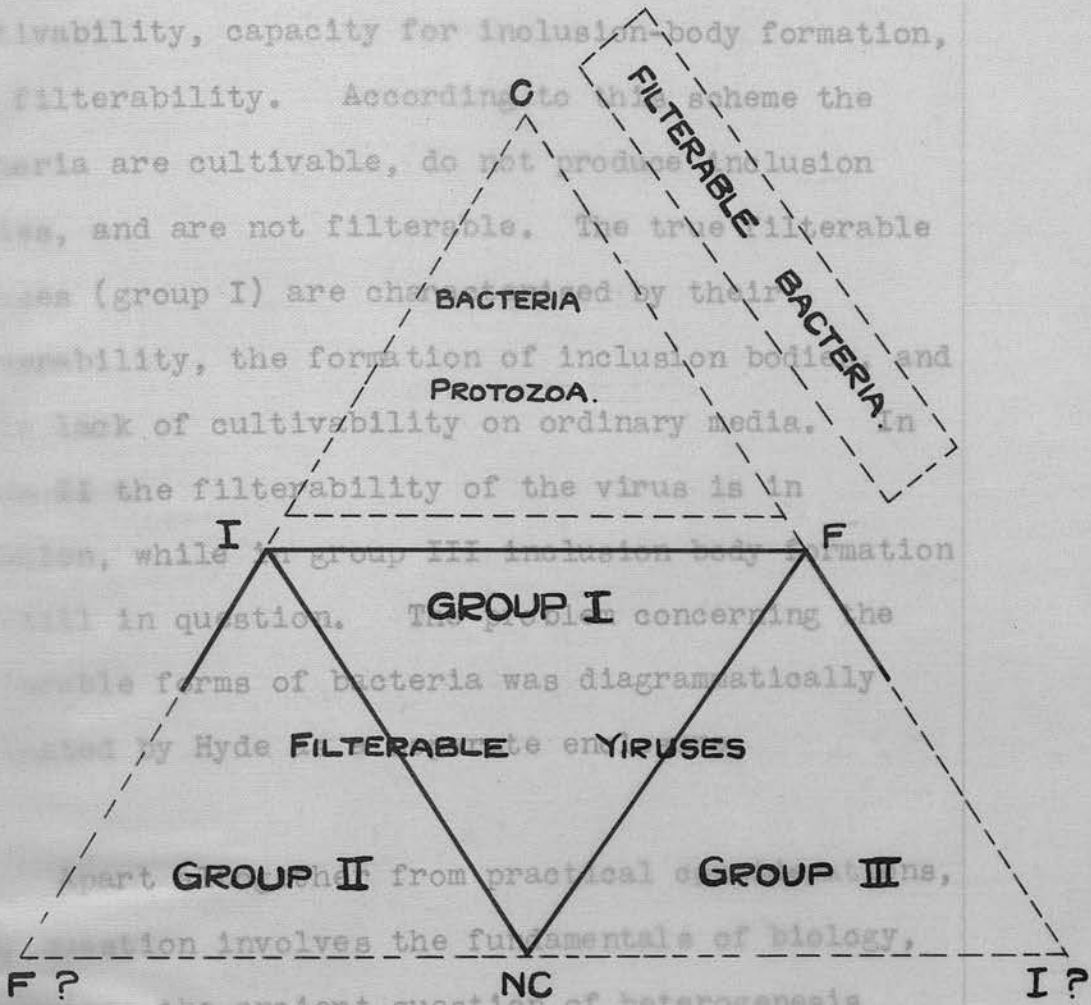
Lastly, many workers, including Calmette, have believed in the existence of a pathogenic "virus form" derived from the tubercle bacillus. The evidence in support of such a view is however incomplete, and in particular the question of filterability has aroused considerable adverse criticism (e.g., Walker and Sweeney 1934). On the other hand, Kuhn (1932) believed that the tubercle bacillus occurred also in a

coccoid, non-acid-fast, granular, so-called 'C-form', identical with some of the Gram-positive granules described by Much, and Fontes (1932) believed that a lipolytic agent in tuberculous pus disintegrated such organisms with the production of a true virus stage. Reynes (1933) concluded that the filterable phase of the tubercle bacillus is not a simple developmental stage but rather "une souche nouvelle" which it is not always possible to obtain from a given culture. In the opening paper of the Ninth Conference of the International Union against Tuberculosis at Warsaw in 1934, Karwacki (for abstract see McLachlan 1935), discussed the question of the biological variations of the tubercle bacillus. The acid-fast organism he believed represented only one phase in a variety of forms which the tubercle virus might assume. The phases described ranged from a filterable and ultra-microscopic stage to a streptothrix, the intermediate forms consisting of granules of varying size and of acid-fast and non-acid-fast bacilli. Thus the tubercle bacillus might be transformed into non-acid-fast or "cyanophil" granules (the "Tuberkelsplitter" of Spengler), into diphtheroid bacilli, into filterable elements, or into a streptothrix showing obvious antigenic relationship as judged by complement-fixation tests. The granular forms were found to be produced under the influence of various dysgenic factors and to be characterised by their marked powers of heat-

resistance. Such forms were recovered from sputa heated at 60° C.-70° C. for periods from one to five hours, and they occasionally survived heating at 90° C. for as long as one hour. In pure culture they produced a "dew-drop" colony on coagulated egg medium, and in animal experiments manifested extremely variable pathogenicity.

From the examples already given it is clear that we must entertain at least the possibility that certain pathogenic organisms, including certain within the ambit of the filterable viruses, are genetically related to and derived from larger bacterial forms of which they are but physiological variants. It is of some interest to note that MacFadyean (1889) observed the small cocci produced by Streptothrix actinomyces invading the animal cell and sometimes even the nucleus and developing therein. Similarly, the gonidia of spirochaetes were seen by Balfour (1911) to behave in the same manner.

In an interesting and duly critical review of the state of knowledge of the filterable viruses, Hyde (1935) indicated the possibility of a relation between this group and the filterable forms of bacteria. Fig. 60 (after Hyde) indicates the relationship between the filterable viruses and the microscopic forms of parasitic life with respect to



C : CULTIVABLE.

I : INCLUSION BODY FORMATION;

F : FILTERABLE;

NC : NOT CULTIVABLE.

(AFTER HYDE 1935)

cultivability, capacity for inclusion-body formation, and filterability. According to this scheme the bacteria are cultivable, do not produce inclusion bodies, and are not filterable. The true filterable viruses (group I) are characterised by their filterability, the formation of inclusion bodies, and their lack of cultivability on ordinary media. In group II the filterability of the virus is in question, while in group III inclusion body formation is still in question. The problem concerning the filterable forms of bacteria was diagrammatically indicated by Hyde as a separate enclosure.

Apart altogether from practical considerations, this question involves the fundamentals of biology, and raises the ancient question of heterogenesis in a modern form. The feasibility of heterogenesis was discussed and admitted by Huxley in his presidential address to the British Association at Liverpool in 1870\*, and it is of the greatest

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\* T.H. Huxley. Presidential Address. In Report of the Fortieth Meeting of the British Association for the Advancement of Science held at Liverpool in September 1870. London 1871.

"But Redi also thought that there were two modes of Biogenesis. By the one method, which is that of common and ordinary occurrence, the living parent gives rise to offspring which passes through the same cycle of changes as itself ---like gives rise to like; and this has been termed Homogenesis. By the other mode, the living parent was supposed to give rise to offspring which passed through a totally different series of states from those exhibited by the parent, and did not return

interest that Dale (1935) has recently revived the discussion in specific relation to viruses. Impressed by the facts that centrifugalisation and ultrafiltration show most viruses to be particles of ascertainable size, that ultraviolet micrography demonstrates their organisation and powers of division, and that immunological methods indicate their specific antigenic composition, Dale inclined to the view that all members of the virus group are micro-organisms multiplying homogenetically and that none are the outcome of heterogenesis. At the same time he admitted that the extracellular virus particle might possibly be a fragment of a virus unit, capable under favourable conditions of regenerating the vegetative form, a conception supported particularly in the case of the psittacosis virus. While such a relatively orthodox viewpoint is probably the correct one in any interpretation of the majority of filterable viruses, it should be pointed out that no available criteria can exclude the possibility of heterogenesis in these specific instances, already discussed, where

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Footnote, continued.....

into the cycle of the parent: this is what ought to be called Heterogenesis, the offspring being altogether, and permanently, unlike the parent. The term Heterogenesis, however, has unfortunately been used in a different sense, and M. Milne-Edwards has therefore substituted for it Xenogenesis, which means the generation of something foreign."

A D D E N D U M

Since the above was written there has appeared an important paper, (D.C.B. Duff, Jour. Bact., 1937, 34, 49-69), describing dissociation in Bacillus salmonicida with special reference to the appearance of a G form of culture. During the course of dissociative activity in serial lithium chloride broth cultures, Duff found that a variant appeared corresponding to the G type culture of Hadley, Delves and Klimek. The same G-variant was also recovered from apparently sterile tubes in the lithium chloride broth series by means of the Hauduroy washed-plate technique. Selected G-strains, when subjected to prolonged serial transfer in lithium chloride broth and in mould-filtrate broth, gave rise to larger colonies, cultures from which proved identical with the known R form of B. salmonicida. These rough non-pathogenic strains later gave rise to typical smooth and pathogenic strains. Of 22 stock G-strains maintained on nutrient agar, 3 reverted spontaneously to the R form after 12 months.

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the known facts are sufficiently suggestive. Notwithstanding Dale's essentially conservative comment, the mere fact that heterogenesis in relation to viruses is admitted as a modern topic can only be construed as a biological sign of the times.

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R E F E R E N C E S

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R E F E R E N C E S

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S U M M A R Y.

In 1931 Hadley, Delves and Klimek described a filterable variant of B. dysenteriae Shiga which was distinguished by individual morphological and cultural characters and which they termed the G-type. Since that date a number of papers have appeared confirming their discovery either in whole or in part. The observations recorded in the present thesis are in almost perfect agreement with the statements of Hadley and his co-workers, although it must not be assumed that agreement extends to the details of interpretation.

The G organism ---derived in the present case from B. paratyphosus B (Tidy) --- proved to be a Gram-negative coccus growing only very slowly in broth and giving rise to minute colonies on agar; differing markedly from the parent culture in biochemical properties; relatively though variably heat-resistant; avirulent and non-toxic; and resistant to a strain of bacteriophage active against B. paratyphosus B.

Over a considerable period of time the G organism tended to give off Gram-negative bacillary variants, all of which showed close biochemical relationship to the G form and to one another. No reversion occurred to the parent form.

These features --- and others --- are discussed in detail in relation to the published work of Hadley and other authors. The discussion also includes an appraisal of the significance of these findings for the homogamic theory of the origin of bacteriophage (Hadley), and a section on the possible relation of filterable forms of bacteria to the problems of communicable disease.

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