THE LIFE-CYCLE OF BACTERIA.

ALTERNATE ASEXUAL AND AUTOGAMIC PHASES.

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(With 11 Figures.)

CONTENTS.

PAGE

Introduction and acknowledgment 379

Sect. (1). Summary of the facts concerning the life-cycle of bacteria and their variations 380

I. Life-cycle. Colony formation, sporing, papilla formation 380

II. Variation in bacteria 382

A. Rare variation or mutation.

B. Variations which occur frequently, and regularly under certain definite conditions.

(1) Changes in "sugar" reactions.

(2) Morphological variations.

(3) Variations in vitality in culture.

III. Unfolding in colony growth of variations which began at the critical phase but were at first latent. Reversionary variation 384

IV. Two factors are required for the primary variation, one external, viz. the stimulus of the appropriate "sugar," and one internal, viz. the critical phase. The first must be applied during the occurrence of the second in order that it should be effective 385

Sect. (2). Hypotheses 386

I. Previous theories of variation in bacteria 386

II. Hypothesis of a life-cycle with alternate phases of asexual reproduction and autogamy, adaptive Mendelian variation at the latter . . . 387

Sect. (3). Additional literature 390

Sect. (4). Additional experiments 391

I. Descendants of young white papillae of *B. coli mutabile* on lactose. Unfolding in asexual multiplication of a variation begun at autogamy . . 391

II. Experiments to prove that more than one factor is required to cause the adaptive variations of coliform bacteria 391

A. A bacterium which varies by papillae to a given sugar will not do so if it is subcultured daily. It can thus be kept in contact with the appropriate sugar for an indefinite period without variation since the critical phase is not allowed to occur.

B. Experiments to show that lactose added during the critical phase to colonies of *B. coli mutabile* growing on sugar-free plates is able to cause variation after a very short period of action.

Introduction and Acknowledgment.

In two recent papers (Stewart, 1926, 1927) the present writer has proposed an hypothesis dealing with the life-cycle and variations of bacteria. Some additional work has been done on the subject in this laboratory since the

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last publication, and as the matter is somewhat obscure and involved it seems desirable in the present paper to summarise all the facts, then to state the various hypotheses which have been framed and to discuss them, and finally to give the new evidence in detail. The previous literature has been dealt with in the two papers referred to and will only be mentioned as necessary in the discussion, but a full list will be given at the end of this paper. One important paper has however been overlooked, viz. Ledingham (1918), and this will be summarised in Section (3)[1].

I wish here to acknowledge my indebtedness to Mr W. F. Gifford, senior assistant in this laboratory, for the greatest help in the course of a long investigation.

SECT. (1). SUMMARY OF THE FACTS CONCERNING THE LIFE-CYCLE OF BACTERIA AND THEIR VARIATIONS.

I. Life-cycle. Colony formation, sporing, papilla formation.

If any bacterium is sown in the usual way from a fluid suspension on to a plate of suitable culture medium, colonies arise from the multiplication of single bacteria. These colonies grow at their margins at a rate roughly constant for the species. When the colony has reached a certain size, which is again roughly constant for the species, it ceases to grow or continues growing very slowly. Thus for example colonies of streptococci grow to 1 mm. in diameter, those of coliforms to 3 or 6 mm. The colonies do not continue to grow until they have covered the whole plate. This limitation of growth is not due to the using up of the food supply or to the drying of the medium, since, if a second sowing of the same bacterium is made between the arrested colonies, a new crop of colonies arises.

In sporing bacteria spores are formed after the colonies have grown for a certain definite time. Thus in *B. tetani* spores appear between 36 and 48 hours; in a race of *B. flexus* isolated in this laboratory they began to appear after 16 hours' growth.

Certain large sporing bacilli, *B. Bütschlii*, *B. sporonema* (Schaudinn, 1902, 1903) and *B. flexilis* (Dobell, 1908), before sporulation, go through a process resembling conjugation between sister cells (autogamy). Thus *B. sporonema* divides by a transverse partition, the partition is then reabsorbed and a single spore or zygote is formed at the junction of the two halves. *B. Bütschlii* forms a similar transverse partition which is also reabsorbed, the chromatin granules arrange themselves in lines along the length of the bacillus, and those of the two halves are mixed by vigorous streaming movements; they then arrange themselves in a longitudinal spiral at each end of which a spore arises. *B. flexilis* divides for a time incompletely into two, the chromatin granules form a longitudinal spiral with bulbous swellings at either end in which two spores arise. In *B. spirogyra* the chromatin granules are permanently arranged

[1] See also Hort, E. C. (1916, 1917, 1917 b, 1920) and Mellon, R. R. (1925, 1925 b), who describe zygospores in colon-typhoid bacteria including *B. coli* mutabile.

in a longitudinal spiral, spore formation is preceded by transverse division and separation of the two halves; one end of the spiral in each sister cell enlarges to form the spore. The sporing forms are always one-half the length of the vegetative forms.

Schaudinn interpreted the phenomena in *B. Bütschlii* and *B. sporonema* as autogamic conjugation. Dobell on the other hand regarded them as abortive division into two which is completed in *B. spirogyra*.

In the race of *B. flexus* referred to above, when the bacillus is about to sporulate, a minute vacuole appears at its centre; thereafter a large oval vacuole is formed in each half of the cell, in which the spores arise. Spore bearers are smaller than the vegetative cells.

It may be argued that if large and highly organised bacteria conjugate as sister cells, then more simple bacteria may conjugate between the halves of an apparently undivided cell, which would then divide to two cells of half size. It would not be possible to see such a process under the microscope, but if other and indirect evidence were to suggest strongly that it took place, we would not be justified in denying its occurrence for this reason.

In both sporing and non-sporing bacteria papillae form on the colonies about the time when the latter cease growing. They never arise at the growing margin. In their earliest stage they are minute hemispherical projections on the surface of the colony and measure 0·05 mm.; they are conspicuously bright and shining by transmitted light, and may look like small air-bubbles. The great majority of these papillae do not grow larger than 0·1 mm., but when any favourable variation arises in them they may grow to a considerable size. They consist essentially of daughter colonies growing out of the parent colony, and in some species such as *B. anthracis* tertiary colonies develop on these secondary ones.

In sporing bacteria the papillae arise from the spores. Preisz (1904) showed that asporogenic races of *B. anthracis* form no papillae; while in sporing strains heating the plate to 65° C. for one hour does not prevent their appearance; and strains which spore early form papillae early as well. In *B. flexus* spores appear at 16 hours, when the colonies measure 3 mm.; while the first papillae can be seen at 46 hours, when the colonies measure 3–7 mm.

Any given strain of bacterium forms papillae when the colonies have been growing for a roughly definite time. They are formed earlier however on the small colonies of a crowded plate than on the larger colonies of a sparsely sown one, in fact the more limited is the growth of the parent colony by so much the earlier will the papillae or daughter colonies arise. This rule can be demonstrated in its extreme form by placing on a culture plate a large drop of a thick bacterial suspension which is allowed to dry without being spread; the plate is then incubated, and a large compound colony appears in 5–6 hours in which the ordinary asexual multiplication of the bacteria is rigorously limited by the pressure of their neighbours. Under these conditions papillae arise very early. Thus one race of *paracolon* which normally formed

papillae at 96 hours now formed them at 46, another at 27 hours instead of 72, another at 46 hours instead of 72, and another at 20 hours instead of 40.

To sum up, bacterial colonies arise by luxuriant but self-limited asexual multiplication; they form daughter colonies or papillae which in sporing species arise from the spores. (For the present I will refer to the events which initiate the spore and the daughter race as *the critical phase.*) The critical phase has been seen only in certain highly organised bacteria, and in these it resembles autogamic conjugation; it occurs on the downstroke of the wave of asexual growth, and if the asexual phase is curtailed then the critical phase is brought on earlier.

II. Variation in bacteria.

Variation occurs in bacteria in two modes: A. Rare variation or mutation; B. Frequent and regular variation, or the adaptive type of Mendelian variation.

A. *Rare Variation or Mutation.*

These variations have been studied most closely in regard to the "sugar" reactions of coliform bacteria. A *paracolon* bacillus if it is grown on a lactose plate forms white colonies with minute colourless papillae. Under ordinary conditions these remain small and colourless, but in a few strains out of many, if the bacterium is allowed to grow on one lactose plate for a month or more, red lactose fermenting forms may appear in the papillae. These rare changes are mutations. They will not be discussed further here (see Stewart, 1926).

B. *Variations which occur frequently, and regularly under certain definite conditions.*

(1) *Changes in "sugar" reactions.*

If *B. coli mutabile* is grown on a lactose plate with neutral red as indicator, it forms white colonies on which, after two or three days, minute colourless papillae appear. After a further interval these papillae become red from the centre outwards; they then enlarge and grow out over the parent colony as victorious daughter colonies. If subcultures are made from young white papillae, the new colonies are at first white, but after 36 or 48 hours sectors of red colour appear in them. Similar subcultures from older red papillae give colonies of two separate kinds, white and red. Thus the daughter race or papilla consists of two subraces, one non-lactose fermenting like the parent, the other a lactose fermenting variant. This variation takes place only in the presence of lactose, and only in the descendants of a papilla. The white subrace goes on forming red papillae on lactose, but the red subrace breeds true as a lactose fermenter even after prolonged growth on non-lactose media.

Similar variations take place in some strains of this "species" in regard to other sugars, and in other species to various sugars. Thus some strains of *B. coli mutabile* and of *B. paracoli* will vary to saccharose or dulcite; *B. ty-*

phosus varies to dulcite; *B. dysenteriae* Sonne to lactose and saccharose; *B. dysenteriae* Flexner to maltose and saccharose. In all cases the variation takes place whenever the bacterium is grown on the appropriate sugar and only in papillae.

A particular strain has its own particular capacity for variation. Thus one *B. coli mutabile* may vary to lactose and dulcite but not to saccharose, or it may vary to all three, or to lactose only. *B. typhosus* varies to dulcite but not to lactose[1] or saccharose. *B. dysenteriae* Flexner (in the great majority of strains) will not vary to lactose.

It is as if each race had a particular setting of instability in its organic constitution, by which it will respond to one particular stimulus whenever that is presented. But once the variation is achieved the variant is stable in that character.

It will be seen that the variation dates from the critical phase, but that it remains hidden and does not become apparent until many generations of the daughter race have been formed. This highly important assertion rests on the fact that subcultures from the youngest papillae will ultimately give red variant colonies without further papillation, but that subcultures from any part of the body of the colony will never do so.

(2) *Morphological variations.*

(*a*) *Capsule formation.* Some strains of naked coliforms will form capsulated variants in the papillae when they are grown on certain sugars. Thus one strain of *B. coli mutabile* did so on dulcite. The variant always capsulates on dulcite, and on dulcite only, and remains true in this character after long growth on non-dulcite media. Here again the variation occurs only in response to one stimulus and only in the papilla, but it takes place whenever the two factors of the stimulus and the critical phase are combined, and again the variants are stable.

(*b*) Changes of many different kinds take place in the shape of the bacteria after papillation in many different species. Thus Preisz, working with *B. anthracis*, found marked differences in the morphology of the bacteria belonging to the primary and secondary colonies.

(3) *Variations in vitality in culture.*

Bernhardt (1915) found that the descendants of papillae of meningococci were more readily cultured and longer lived than the parent strain.

Atkin (1926) found that the pneumococcus, when grown on serum agar, tends to autolyse rapidly. Papillae form on autolysed colonies and subcultures from these papillae no longer autolyse and are no longer soluble in bile[2].

[1] Twort (1907) obtained lactose fermenting variants of *B. typhosus* after prolonged growth in this sugar. These variations do not however occur frequently and regularly. They are probably examples of mutation from a homozygous strain (Mode A above).

[2] The variations mentioned take place in culture where their origin from papillae can be seen. It is likely that exaltation of virulence is an adaptive variation of the same kind occurring in a host.

Having described some of the variations which take place in papillae it is important to repeat that in the great majority of papillae or daughter races no variation can be recognised. Thus even *B. coli mutabile*, which is so highly variable when exposed to certain sugars, still forms papillae on sugar-free media. These papillae remain of small size. *Paracolon* and *colon* also form papillae which do not enlarge, and some spore bearers do likewise.

Therefore the critical phase is not the same as variation. It may be argued that some unrecognised variation may have taken place, but as these variations appear to be all adaptive and therefore beneficial, it is highly unlikely that any such change has occurred in papillae which do not enlarge, and these constitute the great majority of daughter colonies.

III. Unfolding in colony growth of variations which began at the critical phase but were at first latent. Reversionary variation.

We have seen above that in *B. coli mutabile* the variation from white to red on lactose takes origin at the critical phase, but that it does not become apparent until after the papilla has grown to some size, that is until a number of generations have been formed by simple multiplication; for the papilla remains white for one or more days. The process by which the variation unfolds itself can be studied most readily by making subcultures from young white papillae on to appropriate sugar media. In such subcultures the colonies are white, but their centres are accurately divided into a golden half and a white half when viewed by transmitted light. As growth proceeds the golden half becomes red, and we now have particoloured colonies, half red half white, the line of separation being a diameter of the colony. Further cultures from the white half give white colonies only, and from the red half red colonies. In addition to the particoloured colonies, the following types also appear in the subcultures from young papillae, viz. whole white and whole red, but the latter do not become red in less than 36 hours. This unfolding of the variation takes place even if the young papilla is not subcultured directly on to lactose; if for instance it is inoculated into bouillon or into a series of bouillon tubes, and is then plated on lactose, the unfolding is resumed as if the bouillon stages had been left out. It would appear that an incomplete change from white to red has taken place at the critical phase, and that, as the incompletely changed bacteria continue to divide, they separate into two subraces, in one of which the change is completed, and in the other of which it is lost but that this separation takes place only on the appropriate sugar. There is no doubt that even the particoloured colonies are descended from one single bacterium. We are not dealing merely with the separation of two already existing elements of a mixture.

It is important to notice that both the primary variation from white to incomplete "red," and the reversion from incomplete "red" to white, take place under the same external conditions while the bacterium is exposed to the sugar.

Regular reversion. It has been said above that the red race formed by *B. coli mutabile* never reverts to white, but in some other species the red does regularly throw reversionary white forms. Such a race was found in a particular strain of *B. dysenteriae* Flexner (race "A.K.") isolated in this laboratory, the history of which is as follows: When grown on maltose plates it forms white colonies; after 48 hours minute white papillae appear which turn pale red in the next 24 hours. When these papillae are subcultured on to maltose they give colonies of two kinds, white and pale red. As growth proceeds, sectors of two different kinds appear in the pale red colonies, viz. white and dark red. The dark red sectors give dark red colonies in subculture, and the white give white with pale red papillae. Even the dark red colonies continue to form white sectors. In this bacterium then the incomplete change which takes place at the critical phase is only partly completed at the separation of the pale red from the white subraces, it is completed to a further stage when the dark red subrace is formed; it is however never fully completed, so that even the dark red can throw reversionary white. It should be emphasised that there are only three discontinuous shades of colour in this group, white, pale red and dark red. There is no continuous transition of deepening colour from the first to the third.

It should also be emphasised that both the primary variation from white to pale red, the reversion to white, and the progression to dark red, take place under the same external conditions, while the bacterium is grown continuously on maltose; but only the primary variation occurs in a papilla, the other two take place in simple fission in the body of the colony. Reversion however also takes place on non-maltose media.

IV. TWO FACTORS ARE REQUIRED FOR THE PRIMARY VARIATION, ONE EXTERNAL, VIZ. THE STIMULUS OF THE APPROPRIATE SUGAR, AND ONE INTERNAL, VIZ. THE CRITICAL PHASE. THE FIRST MUST BE APPLIED DURING THE OCCURRENCE OF THE SECOND IN ORDER THAT IT SHOULD BE EFFECTIVE.

Everyone who has worked at the variation of coliforms in sugar reactions agrees that variation takes place only if the stimulus of the appropriate sugar is present.

The following experiments show that the stimulus to be effective must be applied at, or shortly before as well as at, the critical phase.

(1) If papilla formation is prevented by daily subculture no amount of exposure to the appropriate sugar will cause variation. Races of *B. coli mutabile*, if allowed to grow on one plate, form papillae on the third day which go red on the fourth. These same races have been kept in daily subculture on lactose plates for 20, 16, and 12 days without any change of colour. Apparently they could be kept so indefinitely.

(2) If the critical phase is brought on early as described above by planting a large drop of thick bacterial suspension on a plate, a mutabile which normally does not bear red papillae until the fourth day now bears them at

46 hours; a *paracolon* which normally bears red papillae on saccharose at 40 hours now bears them at 20.

(3) If a *mutabile* which bears red papillae on lactose on the fourth day is sown on a sugar-free plate, it forms minute colourless papillae from the third day onwards, showing that the critical phase has begun. If now lactose is added to the medium and after $7\frac{1}{2}$ to 24 hours subcultures on to lactose are made from the earliest stage of papillae, then, in some cases but not in all, red colonies will result as well as white. These experiments and others of the same kind show that prolonged exposure to the appropriate sugar before the critical phase is not necessary.

SECT. (2). HYPOTHESES.

I. Previous theories of variation in bacteria.

Theories concerning the variations in sugar reactions of the coliform bacteria have been formulated by Twort, by Neisser and Massini, and by Burri.

Twort considered that the variations were gradual and consisted of the selection in many successive generations of individuals which had a slightly greater fermenting power than their predecessors. This opinion was based on pioneer work with fluid cultures only. When the changes are studied on solid media on which the strains can be accurately analysed, we see that they are not gradual and progressive, but discontinuous and abrupt.

Neisser and Massini regarded the variations as mutation in the sense of De Vries called out by the stimulus of the sugar; and Burri as an adaptation of a special kind, in which a dormant fermenting faculty was activated by exposure to the sugar.

These four workers considered that one factor only was required to cause the variation, namely, prolonged exposure to the sugar. Now we have seen that this factor alone does not cause variation, since in daily subculture a variable bacterium can be kept exposed to the sugar almost indefinitely without change of colour; and on the other hand, if the sugar is applied at or shortly before the critical phase, then the length of exposure may be reduced to one-twelfth or less of that which appears necessary when the bacterium is grown on the sugar in the ordinary way. Also variations of two opposite kinds may take place on the one stimulus, viz. from white to red, and from red to white (as in *B. dysenteriae* Flexner, race "A.K." on maltose).

Nor do these theories afford any explanation of the minute papillae of stable races and of unstable races in the absence of the stimulus, nor of course do they concern themselves with the growth and arrest of bacterial colonies, with sporulation, or with the autogamic conjugation observed by Schaudinn.

It is therefore desirable to look for some hypothesis which will cover all the facts, and which will at the same time be in accord with what is known of other living beings. Such an hypothesis is the following.

II. HYPOTHESIS OF A LIFE-CYCLE WITH ALTERNATE PHASES OF ASEXUAL REPRODUCTION AND AUTOGAMY, ADAPTIVE MENDELIAN VARIATION AT THE LATTER.

(1) Bacteria go through a life-cycle composed of two phases, one asexual, in which the race, if placed in favourable surroundings, multiplies rapidly by simple fission. When this phase is coming to a close the second phase, of autogamic conjugation, sets in, and from individuals which have conjugated there result spores and daughter races or papillae.

(2) At conjugation variations of modified Mendelian type may take place in response to certain external stimuli.

Before we apply this hypothesis to the facts described above it will be well to go very briefly over the life history of a protozoan such as *Paramoecium aurelia* in which conjugation can be seen. If this infusorian is placed in a watch glass of suitable fluid, it multiplies by simple fission, at first slowly, but later with increasing rapidity, the fission rate rising like a wave to a summit and then falling. As this wave of asexual multiplication subsides, individuals begin to conjugate, and as the number of conjugants increases simple fission comes to a standstill. Conjugation can be postponed by daily subculture, by which means the protozoa can be induced to continue asexual multiplication almost indefinitely.

In bacteria we find the same wave of asexual multiplication in colony growth, which is also self-limited unless the race is subcultured. On the down stroke of the wave spores and papillae appear, preceded, in some forms at least, by a process resembling autogamic conjugation. It is hardly necessary to dwell further on the resemblance.

Turning now to the variations, it is suggested that they are of Mendelian type, although they are caused by a stimulus applied at the moment of segregation and conjugation. In *B. coli mutabile* and other races which vary regularly in their sugar reactions we have an unstable non-fermenting parent form which regularly gives off two daughter races, one like itself, and the other stable and fermenting. We know of no biological conception which will cover such a case except that of a heterozygote giving off a homozygous recessive. The corresponding homozygous dominant is found in *B. paracoli* which has been obtained occasionally under unknown conditions from *B. coli mutabile*. It is possible that if we knew the appropriate stimulus we could obtain this form regularly also (see Sect. (3), p. 390).

My meaning will be made clear by a diagram. The Mendelian formula of *B. coli mutabile* is **FFIi**, **F** being a factor for lactose fermentation, **I** a factor for its inhibition, and **i** the recessive absence of **I** (Fig. 1). At the pre-critical division the allelomorphs duplicate, and one set travels into each sister cell (Fig. 2). Segregation then takes place (Fig. 3), followed by conjugation. If lactose is not present the allelomorphs reunite in the parental heterozygous pattern as in Fig. 1; but if lactose is present, then the two recessive **i** factors come together, and the **I** factors are apparently dissipated (Fig. 4).

This scheme is Mendelian in that the unit characters are based on allelomorphic couples. It differs from the Mendelian scheme of higher forms in the following points.

(1) Variation in bacteria takes place in response to an external stimulus and in adaptation thereto, while in higher forms it is due to the segregation of allelomorphs in gametes and their reunion under the laws of chance.

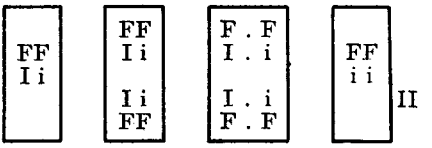

Fig. 1 2 3 4

(2) The variations in bacteria are not completed at the moment of supposed conjugation but require numerous subsequent asexual divisions to become apparent, while regular reversion may take place from an alleged homozygous recessive.

We will consider these points in succession.

(1) Bacteria and higher forms differ in their mode of variation because their mode of conjugation differs. In higher forms conjugation takes place between isolated gametes, each bearing one-half of the allelomorphs of its parent. The male and female gametes unite according to the laws of chance because there is no special affinity between any set of male and any set of female gametes. Let us assume that special affinity does exist between particular pairs of allelomorphs, *e.g.* in the unit character **Aa** there is greater affinity between the factors **A** and **a** than between **A** and **A**, or between **a** and **a**. But **Aa** is only one out of thousands of unit characters in the race and the chance is very slight that any one male and any one female gamete would contain the special affinity allelomorphs of all or of the majority of unit characters. Therefore special affinities, even if present in allelomorphs, would cancel out in gametes, and the Mendelian ratio for unions would remain unaffected. In bacteria, on the other hand, conjugation is autogamic and at the stage represented in Fig. 3, just before conjugation, the four segregated allelomorphs for any one unit character are enclosed within one cell membrane, although they belong to two sister cells. And in their union they are independent of the allelomorphs for other unit characters (since we do not find linked variation even in closely allied unit characters). They can therefore unite according to their special affinity, and if the external conditions have not changed this affinity will be in the same pattern as before segregation. Thus a white *B. coli mutabile* will continue to form white on non-lactose media. If on the other hand a new stimulus has appeared in the surroundings, the special affinity may be reversed and adaptive variation may result, as when *B. coli mutabile* on lactose forms the lactose fermenting colon bacillus (Fig. 4).

(2) The second point of difference probably depends on differences in organisation of the chromatin. In higher forms each factor is concentrated

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on one locus in a chromosome. In bacteria well-defined chromosomes have not been found nor anything resembling a karyokinetic figure, and in the pre-sporing (autogamic) divisions described by Schaudinn and Dobell the chromatin is represented by a vast number of the most minute granules. It is therefore likely that a factor is not represented only at one locus, but is fractionised and distributed through many granules. Therefore Bateson's theory of the fractionisation of a factor and its unit character may be applied freely in bacteria. The following diagrams will explain the matter. A factor is to be regarded not as an indivisible unity but as made up of a number of fractions. We will represent the **I** factor diagrammatically as divided into four, and for the sake of simplicity will omit the fraction signs (Fig. 5). After the pre-critical division (Fig. 6) each factor segregates as before (Fig. 7); and

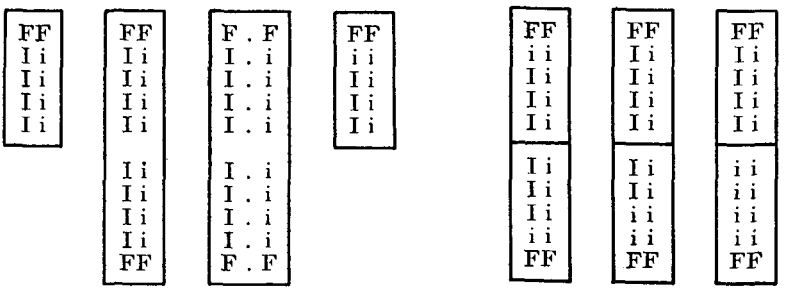

Fig. 5 6 7 8 Fig. 9 10 11

at conjugation in the presence of lactose, only one fraction recombines in the variant pattern, giving the incomplete "red" (Fig. 8). We now assume that in asexual division the fractions duplicate and one-half pass into each daughter cell. If lactose is not present in the medium the variant (recessive) fractions are equally distributed between the two daughters (Fig. 9) and there is no unfolding of the variation, but if lactose is present then the variant fractions tend to congregate in one daughter (Fig. 10). Thus after a certain number of asexual divisions one of the descendants will suddenly emerge as a pure homozygous recessive colon bacillus while its sister cell will revert to pure heterozygous inhibited *mutabile* (Fig. 11). The colony in which this takes place will be particoloured.

We next assume that in some few races the recessive never becomes quite pure but always retains a minute fraction of the inhibitory factor, and so we can explain regular reversion as in the Flexner race "A.K." mentioned above.

To sum up the argument as to the nature of the critical phase, we find that: (1) both critical phase in bacteria and conjugation in infusoria occur on the downstroke of a wave of asexual multiplication; (2) both can be post-poned indefinitely by artificially prolonging asexual reproduction by daily subculture; while (3) the critical phase can be brought on early by artificially limiting asexual reproduction. (4) The critical phase, when it can be seen, resembles autogamic conjugation; (5) it results in the formation of spores and

of daughter races. (6) When a particular stimulus is applied to some bacteria at the critical phase, variations of modified Mendelian character result; the same stimulus applied at any other point of the race history produces no effect. (7) The differences between the variations in bacteria and typical Mendelian variation in higher forms can be explained by observed differences between autogamic and amphigamic conjugation, and between the chromatin organisation of bacteria and higher forms.

SECT. (3). ADDITIONAL LITERATURE.

The following important paper was overlooked in the summary of literature given in my two previous publications. It gives an account of an unusual form of papillary variation, in which a heterozygoate regularly throws the homozygous dominant instead of the recessive as in *B. coli mutabile.*

Ledingham (1918) studied the behaviour of a strain of *B. dysenteriae* Flexner on isodulcite. The original strain when grown in isodulcite peptone water gave full acidity in 24 to 48 hours. When isodulcite plates were spread from these tubes on the third day colonies of two types appeared: (1) colonies which became intensely red in 24 hours and did not form papillae; (2) colonies which did not redden until 48 hours and which formed white papillae on the third day. Subcultures on to isodulcite from these papillae gave a third type; (3) colonies which remained white and formed minute white papillae on the sixth day.

Subcultures from type (1) colonies, both centre and edge, gave type (1) colonies only. Subcultures from the red centres of type (2) colonies gave type (2) and also type (3). (I suggest that the colonies of type (3) may have arisen from the earliest stages of papillae which are not recognisable as such.)

On the hypothesis advocated in this paper I should interpret these facts as follows: type (1) is homozygous uninhibited fermenter, **FFii**; type (2) heterozygous, **FFIi**, the dominance of **I** over **i** is sufficient to prevent reddening until after 48 hours; type (3) is homozygous dominant inhibited, **FFII**, formed by segregation and autogamy in the papillae.

Since type (3) is not found in the original strain it may be assumed that the variation from (2) to (3) takes place only on isodulcite. This variation differs from the great majority of papillary variations in being a negative adaptation instead of a positive (*i.e.* from partial fermenter to complete non-fermenter). It may nevertheless be a true beneficial adaptation. A similar case is recorded by Reiner Müller (1909). This observer grew *B. typhosus* on isodulcite; white colonies with white papillae appeared, and subcultures from the papillae gave colonies more intensely white and of more vigorous growth than their parents. (He also obtained strains which formed red papillae on isodulcite, subcultures from which gave the usual red and white colonies.) Reiner Müller considered that the isodulcite hindered the growth of the bacilli, and that in the white papillae a new type had been formed which had over-

come this unfavourable influence. Ledingham's white strain of Flexner may also be a variant adapted to an unfavourable nidus.

SECT. (4). ADDITIONAL EXPERIMENTS.

I. DESCENDANTS OF YOUNG WHITE PAPILLAE OF *B. COLI MUTABILE* ON LACTOSE. UNFOLDING IN ASEXUAL MULTIPLICATION OF A VARIATION BEGUN AT AUTOGAMY.

If variation really takes place at the autogamy which initiates a papilla, and not in the subsequent growth of the daughter race, it should show itself in subcultures from young white papillae; this should be so even if the subculture is not made directly on to lactose, if for instance a young papilla on lactose is subcultured into bouillon or other lactose-free medium, red colonies should still appear in the next plating on lactose.

Four strains of *B. coli mutabile*, viz. J.R. 508, F.S. 249, I.B. 419, F.M.G. 2357, were grown on lactose plates. Small colourless papillae were picked off on the second or third day, and were either plated directly on to lactose (from a suspension in lactose peptone water) or after 1 or 2 days' growth in bouillon, glucose peptone water, or on saccharose plates. The colonies which appeared on the lactose subcultures were the same in all cases. At 24 hours' growth they were all white, but about half of them showed central masses of more opaque white by reflected light, and of shining golden colour by transmitted light. In some colonies these masses occupied the whole of the centre, but in others they were semicircular in shape and were confined accurately to one-half of the centre. At 48 hours' growth white remained white, but gold had become dark red; and we thus had colonies of three types, white, red, and half red half white. In some cases the red sector took up less than half of the colony but never more. The white and red sectors gave colonies of corresponding colour in subculture. In some few plates from small white papillae a most striking type of colony appeared, viz. white with a central red star.

Paracolon, strain F.S. 3674, which varied to saccharose, gave similar results in subcultures on to saccharose of small white papillae from saccharose plates.

II. EXPERIMENTS TO PROVE THAT MORE THAN ONE FACTOR IS REQUIRED TO CAUSE THE ADAPTIVE VARIATIONS OF COLIFORM BACTERIA.

A. *A bacterium which varies by papillae to a given sugar will not do so if it is subcultured daily. It can thus be kept in contact with the appropriate sugar for an indefinite period without variation since the critical phase is not allowed to occur.*

(Continuation of experiments detailed in *Segregation and Autogamy in Bacteria*, Table XXI.)

B. coli mutabile, strain J.R. 508, on a lactose plate forms small white papillae at 48 hours' growth, which go red at 72 hours. This strain was

subcultured daily for 16 days on a series of lactose plates, the intermediate suspensions being made in lactose peptone water. On the seventeenth day the colonies on the last plate were all white, showing that the bacteria which had been carried on from day to day, and which had been in contact with lactose 17 days, had not varied. These colonies still formed white papillae after 48 hours' growth, *i.e.* on the eighteenth day, which went red on the nineteenth.

B. coli mutabile, strain "I.B. 419," forms papillae at 36 hours which go red at 48 hours. Put through a similar chain of 19 daily subcultures. Colonies on the last plate were all white on the twentieth day, and bore red papillae on the twenty-first.

B. coli mutabile, strain "F.M.G. 2357," forms white papillae after 48 hours' growth which go red at 72 hours. Put through a similar chain of 12 daily subcultures. Colonies on the last plate all white on the thirteenth day; formed white papillae on the fourteenth, which went red on the fifteenth day.

Successive segregation of two multiple factors. (Continuation of *Segregation and Autogamy in Bacteria*, Chap. IX.)

A strain of *B. typhosus* (strain "Bucknall"), when grown on dulcite plates, formed white colonies with pale red papillae. Subcultures from these papillae gave pale red and white colonies. The pale reds formed dark red papillae at 72 hours, subcultures from which gave separate pale red and dark red colonies. It was suggested that this was due to the successive segregation of two multiple inhibitory factors. If this was so, then the pale red form, if kept in daily subculture on dulcite, should not form the dark red variety.

The pale red variety was accordingly put through a chain of 7 daily subcultures. The colonies on the last plate were all white on the eighth, and pale red on the ninth day; there were no dark red colonies even on this the ninth day of dulcite exposure. The expectation was therefore fulfilled. The strain still retained its power of forming the dark red variety when allowed to rest on one dulcite plate for 72 hours and thus to undergo the critical phase, since the pale red colonies on the final plate formed dark red papillae on the tenth day of the experiment.

The case of *B. dysenteriae* Flexner, strain "A.K.," referred to in the paragraph on Regular Reversion, p. 385 above, forms an instructive contrast to this case of *B. typhosus*, "Bucknall." In the former a pale red variety gives dark red and white variants on maltose, but these are formed in asexual colony growth not in the sexually formed papilla. If therefore this pale red variety is kept in daily subculture it continues to form the two variants. They appear in the first 24 hours of the third daily plate.

B. *Experiments to show that lactose added during the critical phase to colonies of* B. coli mutabile *growing on sugar-free plates is able to cause variation after a very short period of action.*

Difficulties of this experiment.

(1) If the lactose is added rudely the phase of conjugation will be dis-

turbed and will cease, and the bacteria will embark on another period of asexual growth. This takes place if lactose solution is poured over the colonies, or if the bacteria, when about to conjugate, are transferred to a lactose plate. To escape this difficulty the following manœuvre was adopted. The bacteria are grown on a plate of sugar-free MacConkey's medium for 3 days or more. Small wells are then cut out in the agar between the colonies, which are then filled with 5 per cent. lactose solution and this diffuses through to the colonies with the least possible disturbance. In estimating the length of time during which a bacterium has been exposed to the lactose it is therefore necessary to deduct several hours for the time occupied in the diffusion of the sugar through the medium.

(2) In picking off bacteria from the colonies after the above manœuvre there is no guide as to where bacteria will be found which have conjugated after the lactose has reached them. The most minute papillae may have originated before this, and the bacteria we are in search of may not have had time to form papillae. To meet this difficulty a large number of the smallest papillae may be picked off, or the centre of the colony may be touched with the needle in the hope of getting recently conjugated bacteria. It will be realised that we cannot expect to succeed in every case under these conditions, more especially as the bacteria taken off must be suspended in about 10 c.c. of fluid and only about 0·1–0·2 c.c. of this is examined by spreading on the lactose plates.

(A) *B. coli mutabile*, strain "F.S. 249," when sown on lactose MacConkey plates, shows early white papillae between 72 and 96 hours' incubation, and red papillae between 96 and 120 hours. The phase of conjugation therefore begins at about 72 hours.

Exp. 1. Bacteria sown on sugar-free MacConkey plates. On fifth day minute papillae were visible; lactose added to the wells; 20 hours thereafter many minute papillae from colonies near the wells were picked off into lactose peptone water in watch-glasses (i) and (ii), and minute papillae remote from the wells into watch-glass (iii). Six drops from each watch-glass were sown on separate lactose plates. After 48 hours' incubation, plate (i) showed 8 red colonies, 2 particoloured red and white, and many white colonies; plates (ii) and (iii) white colonies only. Red colonies from plate (i), subcultured on to lactose, gave red colonies only.

Exp. 2. Sugar-free plates. On tenth day many minute papillae; lactose added; 24 hours thereafter three lactose plates sown, (i) and (ii) from papillae near the wells, (iii) from papillae remote from wells. After 48 hours, plate (i) showed 1 red, 10 white colonies; (ii) 25 red, 80 white; (iii) white only.

Exp. 3. Sugar-free plates. On twelfth day many minute papillae on periphery of colonies, papillae sown on lactose plate (i); lactose then added to wells of sugar-free plate; and 12 hours thereafter peripheral papillae near wells sown on lactose plate (ii), and most minute central papillae on (iii); 36 hours

after the addition of the lactose the sugar-free plate showed 1 red and many white papillae; lactose plate (iv) was now sown from the red papilla and (v) from minute white papillae. After 48 hours' incubation, plates (i) and (ii) showed white colonies only; (iii) 1 red, many white; (iv) red, particoloured, and white colonies in equal numbers; (v) 1 red, 30 white.

Exp. 4. Sugar-free plates. On ninth day many minute papillae; lactose added; 24 hours thereafter the old minute papillae show no change, on colonies near wells new papillae have developed; lactose plates (i) and (ii) sown from apparently old papillae near wells; (iii) and (iv) from new papillae. After 48 hours, plate (i) 5 red, many white; (ii) white only; (iii) 25 red, 100 white; (iv) 1 red, 14 white.

Exp. 5. Sugar-free plate. Lactose added on fifth day, papillae subcultured 24 hours thereafter, only white colonies resulted.

(B) *B. coli mutabile,* strain "J.R. 508," when sown on lactose plates, shows minute white papillae at 48 hours' incubation and red papillae at 72 hours; therefore the phase of conjugation begins shortly before 48 hours.

Exp. 1. Sugar-free plates. Many minute papillae on fourth day. Lactose added on seventh day; 24 hours thereafter minute papillae near wells sown on lactose plates (i) and (ii); centre of colony near wells (which was then apparently papilla-free but which 2 days later was covered with papillae) sown on to lactose plate (iii); edge of colony near wells on to (iv). After 48 hours, plates (i), (ii) and (iv) showed white colonies only; plate (iii) 1 red, 90 white.

Exp. 2. Sugar-free plates. On third day minute papillae around periphery of colonies; lactose added; 4 hours thereafter minute papillae near wells sown on to lactose plate (i); $7\frac{1}{2}$ hours after addition of lactose centre of colony near wells on to lactose plate (ii); 24 hours after addition of lactose centre of colony near wells on to lactose plate (iii); minute papillae near wells on to plate (iv); minute papillae remote from wells on to plate (v). After 48 hours, plates (i), (iv) and (v) white only; plate (ii) 1 red, many white; plate (iii) 1 red, many white.

Exp. 3. Sugar-free plates. Lactose added on fifth day, subcultures from papillae $16\frac{1}{2}$ hours thereafter, white colonies only resulted.

(C) *B. coli mutabile,* strain "F.M.G. 2357," when sown on lactose plates, forms minute papillae at 72 hours, and red papillae at 96 hours' incubation; therefore the phase of conjugation opens at about 72 hours.

Exp. 1. Sugar-free plates. On fourth day many minute papillae; lactose added on seventh day; between 24 and 27 hours thereafter 5 lactose plates sown from minute papillae near wells, all gave white colonies only. Three plates sown 48 hours after addition of lactose, (i) from minute central papillae near wells; (ii) and (iii) from peripheral papillae near wells. After 48 hours, plate (i) showed 3 red, many white colonies; plates (ii) and (iii) white only.

Exps. 2–4. Similar; gave only white colonies.

Thus the shortest gross lactose exposures found effective in causing variation to red were of $7\frac{1}{2}$ and 12 hours, and deducting at a guess 2 hours for the diffusion of the sugar from the wells to the colonies, we arrive at an actual net lactose exposure of $5\frac{1}{2}$ to 10 hours.

These experiments are controlled by the experiments detailed in Sect. (4), II. A, p. 391, and in *Segregation and Autogamy in Bacteria*, Table XXI, in which colonies which had grown for 24 hours on lactose and had not reached the stage of conjugation, gave white daughter colonies in subculture without exception. There were no red colonies even after 20 days contact with lactose.

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