STUDIES IN BACTERIAL VARIATION.

WITH SPECIAL REFERENCE TO THE CHEMICAL FUNCTIONS OF THE MEMBERS OF THE TYPHOID-COLI GROUP.

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With Plate I, and 9 Charts.

MUCH recent work has demonstrated the occurrence of variation in the fermentation properties of intestinal bacteria. As is well known these functions are of great service in the differentiation of species and varieties and accordingly any evidence of instability on their part assumes at once considerable practical importance. These changes may show themselves by (1) the acquirement of a new fermentation character, or (2) by the loss of such a character, or the two processes may go on simultaneously. The acquirement of such a new character may be sudden or gradual.

The sudden changes have received particular attention since M. Neisser (1906) described a case of mutation, in which a certain coliform organism took on the lactose-fermenting power suddenly and retained that power with great tenacity. This organism was subsequently very fully investigated by Massini (1907), who demonstrated convincingly that the new lactose-fermenting organism was in reality derived from the non-lactose-fermenting ancestor and also that it was absolutely stable. On lactose-fuchsin-agar it was found to form white colonies, but from the second day onwards papillae (Knöpfe) developed on those colonies which were not crowded together. The papillae were white at first, becoming red later. They appeared only if milk sugar was present, a concentration of 0·1 % of this sugar being sufficient to

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produce them. Sub-cultures from the white colonies, within 24 hours of their appearance, gave only white colonies on a lactose-fuchsin plate. These colonies remain white, but develop red papillae. Sub-cultures from the papillae, however, give both red and white colonies, and from the former red colonies only appear on sub-culture. Papillae never form on them. The red strain remained constant during many months of culture apart from lactose, and Massini after trying many devices found the new characters could only be removed by growing the organism in carbolic acid media. This fact, however, does not conflict with the idea of the absolute stability of the new character, as the addition of carbolic acid has been shown to destroy the natural fermenting power of many organisms. An antiserum made by immunising with the white strain agglutinated red and white organisms equally highly and a similarly prepared serum for the red organisms acted equally on both.

It appeared, therefore, that a new type of organism had arisen incapable of papillae-formation on lactose but capable of fermenting this sugar.

Subsequently Burk (1908) described a similar organism, but its serological reactions were not quite so satisfactory as in Massini's case. Reiner Müller (1908–1909) described a number of instances in which organisms behaved in an analogous manner, all of which he interprets as being of the nature of mutation. From faeces he isolated two non-lactose fermenters, colonies of which developed red papillae on litmus lactose agar. Sub-cultures from these papillae fermented lactose. Three other strains isolated from faeces formed papillae on arabinose, but further sub-culture on this medium was attended by loss of this papillae-forming power, although a more vigorous growth resulted. One of these three strains in the process of variation lost the power to ferment galactose which it formerly possessed. Fifty strains of *B. typhosus* were found to produce these papillae on isodulcite, and sub-culture from the papillae gave, in each case, colonies (1) capable of papillae-production, (2) incapable of further papillae-production and either able or unable to ferment isodulcite.

B. dysenteriae (Flexner type) behaved in the same way on isodulcite. *B. paratyphosus* (B.) also produced similar papillae on raffinose. Reiner Müller concluded from his work that these mutation-processes are not at all infrequent and that they are specific, *i.e.*, take a definite direction for certain definite well-defined organisms. He worked with many organisms, but not in so thorough a manner as Massini, and in respect of

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constancy of the new types, and absence of atavism, his work affords us no convincing evidence. Such constancy is essential before the process can reasonably be called mutation in the sense of de Vries. The only evidence he gives of mutation, in many cases, is papillae-formation; he does not sufficiently establish the constancy of the new types, and my own work leads me to conclude that the formation of papillae is not sufficient evidence, in itself, of such constancy.

The formation of papillae (Knöpfe) had been described before by Preisz (1904) and Eisenberg (1906). The former noted it in the case of anthrax colonies on ordinary nutrient agar of faintly alkaline reaction. The secondary colonies appear as papillae on the surface of the colony, and are hemispherical, smooth, and whitish to yellow or yellowish brown. They give the appearance to the colony of a foreign organism having grown as a contamination. After weeks or months these secondary colonies may themselves develop papillae, *i.e.*, tertiary colonies.

The anthrax papillae arise from proliferation of spores, since asporogenic races do not give them and heating at 65° C. for an hour does not prevent their appearance. Moreover if the strain is an early spore-bearer, the papillae appear early, and if late, they appear late.

Marked differences are found in the morphology of the bacilli belonging to the primary and secondary colonies. No evidence is given, however, of any new transmissible character having been acquired.

Preisz also describes, briefly, secondary colonies on cultures of *B. diphtheriae*, *B. cholerae* and *V. Finkler-Prior* and attributes them to the selection of stronger and more resistant individuals.

Eisenberg (1906) described granulations on the deep aspect of typhoid, and other colonies, on the addition of egg albumin to the medium. They appear in about two days and are of small size. The early ones coalesce, while the later ones appear near the margin of the colonies. He could not get this appearance on early cultures on ordinary agar, but in old cultures they very occasionally appeared in the case of *B. typhosus* and *B. dysenteriae*. He believes they are due to the detachment from the colonies of bacilli which penetrate more deeply into the medium and start small adjacent colonies. They are moved in the case of motile organisms by their own power and in the case of non-motile, he believes by diffusion. The diffusion of motile anaerobes through agar from a stab culture in the cold room has been demonstrated by Koninski (1902), so that the possibility of such diffusion has to be considered.

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The fact that the granulations might occasionally appear in old agar cultures after some weeks (typhoid and dysentery) but never in young cultures, does not suggest that the granulations are an accompaniment of vigorous growth.

Serkowski (1901) has described daughter colonies but they are simply the cell conglomerates of which he finds bacterial colonies in general are built up. The chemical composition of the medium was not a determining factor. His secondary or daughter colonies have no resemblance to the structures we are dealing with. On the other hand, Eisenberg's granulations may very reasonably be analogous to Massini's papillae in so far as an adaptation to food supply may be the moving factor in each case.

With regard to gradual changes in the fermentation properties of the typhoid-coli group the most interesting results have been obtained by Hiss (1904) and Twort (1907). Twort found he could make *B. typhosus* ferment dulcite and lactose by simply growing it on the carbohydrate he wished it to ferment, for long periods with fortnightly sub-cultures. The method is similar to that of Neisser, but was employed for a much longer period. These sub-cultures were observed for a month. In the same way he caused *B. dysenteriae* (Shiga-Kruse) and *B. dysenteriae* (Flexner) to ferment saccharose, *B. dysenteriae* (Shiga-Kruse) to ferment lactose and the paratyphoid organisms to ferment saccharose.

Twort gives us no idea of the different lengths of time selection had to be carried on to secure his new varieties. There seems to be a good deal of evidence to show that this factor is related to the permanency of the new characters, this information would be of great value.

His successful variations caused him to regard the sugar tests as an impossible means of grouping the intestinal organisms. Reiner Müller, on the other hand, suggests that the power to vary in particular directions must be looked upon as an additional means of identification.

The principle at the basis of these variations is stated by Twort to depend on selection of individuals inclined to use the particular carbohydrate, when the other constituents of the medium are used up. This cannot be the case in the group of so-called mutations, as the change occurs when the medium is still far from being exhausted. Both groups have adaptation to food supply as a common feature, and this factor is not improbably operative in the case of Eisenberg's granulation-

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formers. In the rapid cases, however, the determining cause is almost certainly the presence of inhibiting agents which hinder growth on the particular medium, but do not prevent it so powerfully on a food supply of another character. I shall go into this question more fully at a later date, but the work of Eijkmann (1904) and Konradi (1904) has shown that the arrest of growth in cultures is brought about by inhibiting agents, rather than by exhaustion of food supply. My observations confirm this. Goodman (1908) obtained some results by artificial selection in the case of the diphtheria bacillus, which are worth mentioning. By artificial selection of the highest and lowest acid-formers in a series of dextrose broths, and inoculation of a double series from them, repeating the process 36 times, Goodman obtained a strain producing intense fermentation of dextrose and another strain not fermenting it, but actually rendering the broth more alkaline. The titrations were made in each case after three days' growth at 37° C. In each "higher" series the tube of highest acidity was selected, in each lower series the lowest. He determined the acid-forming power by titration with 1/20 N. soda. It is remarkable that the variation went on in opposite directions about equally quickly. Goodman concluded that the fermenting character is a poor guide to determine whether an organism is pathogenic or merely a harmless saprophyte. The difference he obtained was as great as the natural difference between a *B. diphtheriae* and *B. pseudo-diphtheriae*. Morphological differences between the two were not marked. The greater-acid producing strain grew more vigorously than the lower one on Loeffler's serum, during the first 24 hours. The influence of this selection upon the power to ferment dextrin was negligible, both were diminished in fermenting power for maltose, and in both cases the capacity to ferment saccharose was enhanced.

Pathogenicity tests were few, but such as were given show that the greater the fermenting power, the greater is the virulence. It is remarkable that continuous growth on dextrose should have so readily yielded a strain not attacking it, derived from a strain using it vigorously. The variation in the organism in this case tends in the direction of diminished vegetative activity. Goodman does not state whether on sub-culture without selection the races tended to return to their original characters or not. If in this way he had or had not obtained fair constancy of his races, his results would have been more valuable and complete. This loss of fermenting power so effected, seems

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on the face of it at variance with all experience hitherto obtained in the intestinal group. The logarithmic rate of increase of the organisms in the culture would be pulled up after a few hours' growth, after which one would naturally expect that the dextrose present would lead to a constant selection in favour of its fermenters. An increase of fermenting power in these conditions would thus be anticipated rather than a decrease. Goodman takes no cognizance of Neisser's, Massini's and Twort's work and accordingly volunteered no explanation of this occurrence.

De Vries found that in plants, selection from the extremes of the fluctuating variability of a species will not give rise to a new pure race. It will vary the percentage of plants with the special character, to a certain degree, after which further selection seemed of no value, while *in the cases of mutation, new and constant races arose immediately.*

Of other variations in fermentation power I might add the following:

Hiss (1904) found that the bacillus "Y," a type of *B. dysenteriae,* when cultivated for some time on maltose media, had taken on the power to ferment maltose. This result is confirmed by Lentz and Kutscher (1909). Lentz (1909) also describes a Flexner-strain which in laboratory culture after seven years lost the power to ferment maltose, though still retaining its agglutinating power. Klotz (1906) by passage through an animal, caused an organism to lose a certain fermenting power, but found it was regained by the fourth sub-culture on the particular sugar. The sub-culture lasted only one day. Sub-cultures on ordinary media caused it also to regain the power, only more slowly.

Much old work on variation in fermenting power in the typhoid-coli group is given by Rodet (1894) in his *De la variabilité dans les microbes,* but since he had not the advantage of the agglutination tests one cannot attach much importance to the results. Reliable work of this kind must be done with an organism well defined by many established tests, and evidence of change without these means of identification is almost useless.

Many other functions of bacteria have been made the subject of study in the matter of inheritance, which, since they throw some light on the general aspects of the subject, ought not to be entirely overlooked. Since I have, however, occupied myself mainly with the fermentation characters, I will leave over a consideration of further papers till I endeavour to fit my own findings into the general edifice of modern results on bacterial variation.

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PART I. VARIATION IN THE FERMENTATION CHARACTERS OF THE INTESTINAL ORGANISMS.

B. typhosus and dulcite. *B. typhosus* is usually stated not to ferment dulcite broth. This is due to the fact that only a short period of observation is undertaken. If the observation is continued for two or three weeks at 37° C., full acidity is usually produced, but occasionally the reaction is slight, even after this period, and careful comparison with a control may be necessary to determine it. Fourteen different strains tested in one experiment showed the first signs of acidity, as indicated by litmus, in from five to fifteen days, with an average of nine days (see Table I).

TABLE I.

	"E" 6	"E" 8	"E" 9	"E" 10	Lincoln	"D"	McConn	Gray, F.	"R"	"H"	"W"	King	Guy	Christie
5th day after seeding	—	—	—	—	—	—	—	—	—	—	A	—	—	—
6th do.	?	—	—	—	—	—	—	—	—	—	—	—	—	—
7th do.	A	—	A¼	—	—	—	—	—	A	—	—	A¼	—	—
8th do.	—	?	A	?	—	—	—	—	—	—	—	,,	—	—
9th do.	—	?	—	A½	—	—	—	A½	—	—	—	A½	—	—
10th do.	—	?	—	,,	—	—	A¼	,,	—	A¼	—	,,	—	A¼
11th do.	—	A½	—	,,	A	A½	A½	,,	—	,,	—	,,	—	A½
12th do.	—	,,	—	,,	—	A	A	,,	—	A½	—	,,	—	,,
14th do.	—	,,	—	,,	—	—	—	,,	—	,,	—	,,	—	,,
15th do.	—	,,	—	,,	—	—	—	,,	—	,,	—	,,	A¼	,,

The initial reaction of the medium was in each case twelve and a half degrees acid to phenolphthalein. A = full acidity. The various states of partial acidity are indicated by A plus a fraction, the fraction giving an indication of the extent to which the acidity had advanced.

The previously mentioned strains on dulcite broth were sub-cultured after one month at 37° C., when it was found that acidity was produced in from one to four days—average 2½ days—as shown in Table II.

TABLE II.

	"E" 6	"E" 8	"E" 9	"E" 10	Lincoln	"D"	McConn	Gray, F.	"R"	"H"	"W"	King	Guy	Christie
1st day after seeding	—	—	—	—	—	—	—	A	—	—	—	A½	—	—
2nd do.	—	A	—	A	—	A	—	—	A	A	—	A	A½	—
3rd do.	A	—	A½	—	A	—	A	—	—	—	A	—	A	—
4th do.	—	—	A	—	—	—	—	—	—	—	—	—	—	A½

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One month later the same strains were sub-cultured and all showed full acidity in from one to three days; no less than six of them producing sufficient acid to colour the litmus in one day. (See Table III.)

TABLE III.

	"E" 6	"E" 8	"E" 9	"E" 10	Lincoln	"D"	McConn	Gray, F.	"R"	"H"	"W"	King	Guy	Christie
1st day after seeding	A$\frac{1}{2}$	A	—	A$\frac{1}{2}$	—	—	—	A	—	—	Did	A	—	A
2nd do.	A	—	—	A	A	A	A	—	A	A	not	—	A	—
3rd do.	—	—	A	—	—	—	—	—	—	—	grow	—	—	—

The first seven strains to produce full acidity in the first generation required in the second generation $2\frac{5}{7}$ days, while the seven slow ones in the first generation required only $1\frac{6}{7}$ days in the second generation. This apparent discrepancy arises probably from the injury caused to the organism by the acid produced, which would be greater in the case of the earlier fermenters. It will be seen from the Tables I to III that the first one to produce full acidity in first cultures had died out by the third, and in the second generation caused acidity relatively slowly. As will be seen later, however, at least one other factor influences the period at which sub-culture will give the best result.

Sub-cultures of the first generations of this series of seven were made after 13 days' incubation and these reached full acidity in $2\frac{1}{7}$ days, *i.e.*, distinctly earlier than when sub-cultured after one month.

The behaviour of *B. typhosus* on neutral-red-dulcite-agar was next studied. The typhoid strains of Table I, E 8, E 10, King, Christie and Gray were selected for the purpose as they had all reached the stage of fermenting dulcite broth in one day. For seeding the plates, ordinary broth cultures of the organism, having no previous dulcite inheritance, were used. The results are set forth in Table IV.

The papillae appeared in these strains as early as the third day, and by the fifth day they were acid in reaction. After that time they commenced to fade and took on a dirty yellow colour. The red points that appeared in the centres of the plates on the crowded portions are probably analogous to papillae, as none of the larger colonies took on the acid reaction at any stage of growth.

Quantitative estimation of papillae-bearing colonies in the case of strains Christie, King, and E 10, was made on plates spread from diluted broth cultures. The plates were not at all crowded. On the

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sixth day after plating, wide variations were found. Some plates gave as low as 2 per cent. of these colonies and some as high as 50 per cent. The same strain varied within these limits on different plates.

The absolute number of papillae on the colonies possessing them is usually one to three, but occasionally the number rises to as many as 12 by the tenth day, at which time also the proportion of colonies bearing papillae has usually considerably increased.

TABLE IV.

(See Figures 1 and 2, Plate I.)

Plates inoculated, 25/8/09	E 8	King (W)	Christie	Gray, F.	E 10
Observed 26/8	Typical colourless typhoid colonies	As E 8	As E 8	As E 8	As E 8
29/8	All colonies still without any sign of acid reaction	As E 8	As E 8	As E 8	As E 8
30/8	White papillae appearing on many of larger colonies, one to three on each	As E 8	As E 8	As E 8	As E 8
31/8	Some of the papillae are acid in reaction to-day and some acid points are found on crowded portions of the plates	As E 8, only the acid papillae are few in number	As E 8, only the acid reaction of papillae is not so marked	As E 8	No red papillae yet
1/9	As yesterday	Acid papillae increasing in number	As E 8	As E 8	A faint suggestion of acid reaction obtained in some papillae
7/9	The papillae have now faded and are of a buff colour	As E 8	As E 8	As E 8	As E 8

A papilla on a dulcite plate, put through ordinary broth and plated again on neutral-red-dulcite-agar, gave, in the case of "Gray," 50 % red and 50 % white colonies by the fourth day after plating. By the sixth day some of the white colonies showed papillae while others did not.

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The ratio of red colonies to white, in the case of E 8, E 10, Christie, and King, was different, but otherwise the plates gave essentially the same results.

The same five strains of *B. typhosus* used in the previous experiment were now grown on dulcite broth for two days, and on plating again on dulcite agar gave the result set forth in Table V.

TABLE V.

Plates inoculated, 20/8	Christie	King	Gray, F.	E 8	E 10
Plates observed 21/8	Typical colourless typhoid colonies	As Christie	As Christie	As Christie	As Christie
23/8	Papillae appearing. All colonies colourless	No papillae yet. No acid colonies present	All larger colonies show an acid reaction	As King	As King
24/8	No further change	Papillae appearing	No papillae present	As King	As King
25/8	Papillae are still colourless	Up to seven papillae on one colony	No papillae	No further change	No further change
26/8	Papillae are acid in reaction	As Christie	No papillae. All large colonies smooth and acid in reaction	Papillae acid in reaction	All papillae still colourless
7/9	Papillae faded	Papillae faded	All large colonies have now faded	As Christie	As Christie

Result:—The appearances in the case of Christie, King, E 8 and E 10 were similar to those given in Table IV, where the plates were made from normal broths, but all the larger colonies of Gray were fermenters of dulcite and did not produce papillae, while some small non-fermenting colonies did show them.

After five days' growth on dulcite broth, three of the above strains were plated on neutral-red-dulcite-agar with results given in Table VI.

TABLE VI.

Plates inoculated, 23/8	King	Gray, F.	E 10
Observed 24/8	No acid colonies present	As King	As King
25/8	,, ,, ,,	All larger colonies red	,,
26/8	,, ,, ,,	,, ,, ,,	,,
27/8	,, ,, ,,	,, ,, ,,	,,
1/9	Many acid papillae present in case of larger colonies	No papillae have appeared	,,
10/9	Papillae faded	,, ,, ,,	,,

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The papillae on the white colonies did not come any earlier after the five days' growth on dulcite broth than they did when plated from ordinary broth. Apparently the variation leaves some of the cells entirely unaffected.

After a period of eight days in dulcite broth, strains "Christie," E 8, and "Gray" had all lost the power to form papillae, but had acquired the power of fermenting the dulcite vigorously. "King" and E 10 showed on the other hand a mixture of smooth red colonies and white papillated ones, the papillae being red. The papillae did not appear till the fifth day.

Red papillae on dulcite plates inoculated into dulcite broth only take three days to produce definite acidity, while the average time of the original strains of the same is eight days. Individuals removed from the smooth part of the colony also required eight days.

If papillated colonies be inoculated into ordinary broth and then plated on dulcite agar, red and white colonies result. One of these red colonies requires only two days to produce acidity in dulcite broth. In this case the non-fermenting elements are entirely avoided.

Normal strains of *B. typhosus*, which by the third generation on dulcite broth did not attain the power of fermenting it within 24 hours (Table III), were plated out on neutral red dulcite agar and gave the following result (Table VII).

The observations upon these more slowly varying organisms are set forth in Table VII and may be summarized as follows:

(1) No red colonies produced by any strain.
(2) None which are able to produce red papillae.
(3) Two out of seven not able to produce papillae in the early stages of growth, *i.e.*, within, say, 16 days.
(4) Five able to produce white papillae.
(5) One able to produce late papillae.

This series was then plated out from dulcite broth four days old, and H, which had not produced papillae before, showed papillae on the eighth day, probably because of some variation occurring in the broth. Four of the other strains showed a few red colonies.

The effect of the nutrient agar basis of the medium in producing papillae was tested in the following experiment. Eight strains of *B. typhosus* were inoculated on ordinary nutrient agar plates and observed from September 6th to October 14th, 1909, but no sign of papillae-formation was seen. I have never succeeded in getting papillae on single colonies of *B. typhosus* on ordinary nutrient agar, so

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that we must look upon the secondary colonies on dulcite plates as due to the dulcite, especially since the papillae contain elements capable of fermenting dulcite to an extraordinary degree. It must however be admitted that one sometimes sees papillae on nutrient agar slopes of *B. typhosus*, but the significance of these has not, so far as I am aware, been determined.

TABLE VII.

Inoculated 6/9	R	Lincoln	McConn	H	Guy	D	E 9
Observed 7/9	Typical colourless typhoid colonies	As R	As R	As R	As R	As R	As R
8/9	Typical colourless typhoid colonies	As R	As R	As R	As R	One salmon colony amongst a large number of white ones	As R
10/9	No papillae present. No colonies of acid reaction	As R	As R	As R	As R	Salmon colony faded	As R
13/9	Papillae appearing	All colonies white, and no papillae present	As Lincoln	As Lincoln	As Lincoln	As Lincoln	As Lincoln
15/9	More papillae present, none of them acid however	No papillae present	Papillae have appeared	As Lincoln	As Lincoln	As Lincoln	As Lincoln
16/9	—	Papillae have appeared	—	No papillae present	As Lincoln	Colourless papillae present now	No papillae present
22/9	—	—	—	—	—	—	No papillae present
8/10	—	—	—	—	—	—	Papillae present now

We may, therefore, conclude that the normal *B. typhosus* produces only white colonies on dulcite agar plates, but some of these may get red papillae and at the same time red points may appear on the crowded parts of the plates, these points being equivalent to papillae. I have never seen a large colony of a normal typhoid strain which was red after 48 hours' growth on dulcite-neutral-red-agar. The nearest approach was in the case of "D" (see Table VII). In the course of 46 subsequent plates examined after 48 hours' growth at 37° C. I never observed the salmon-pink tinted colony again.

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Twort trained on dulcite broth and his evidence of variation was the production of acidity in broth. He appears to have been unaware that the majority of typhoid strains naturally produce acidity in dulcite broth. My experience is that every strain will do it without training, though it may be that in the case of some rare strains three or four tubes of the particular strain will be required to demonstrate this fact.

I take it that the strain he trained was peculiarly refractory to dulcite, and this shows us the need for examining a large number of strains. The normal *B. typhosus,* judging by over 20 strains I examined, will not produce acid colonies on non-crowded plates of dulcite agar, but one strain in dulcite broth showed on plating that it contained red elements as early as the second day, and some red colonies were obtained in dulcite broths from two to ten days old in the case of all strains tested, though not from every single colony.

By the simple expedient of colony selection a typhoid strain fermenting dulcite in two days may be obtained after only two days' training on dulcite. See Table V, strain "Gray."

The sequence of events in the process of variation in the case of the "B. typhosus" grown on dulcite broth.

This was studied in the following way:

(1) The total count per c.c. of the broth culture was taken daily.

(2) The ratio of fermenting to non-fermenting (red to white) elements was determined by plating on neutral-red-dulcite-agar daily. The plates were observed after 48 hours' growth at 37° C. in each case. They should have 80—100 colonies on the surface and these should be evenly distributed.

Nine such experiments were carried out.

For the purpose of these experiments strain "D" was selected, as it took about 12 days to produce acidity and consequently gave plenty of time for many observations. Occasionally this strain did not become fully acid and it was therefore considered desirable to follow the behaviour of both these varieties.

There are two main types (see Charts I—VII):

In the first type, *i.e.,* the type to go fully acid, the total count is usually 200 to 300 millions per c.c. during the first 7 to 14 days of growth, then it suddenly rises to 600 and to 1000 millions per c.c. Coincident with this sudden exacerbation of growth the medium becomes

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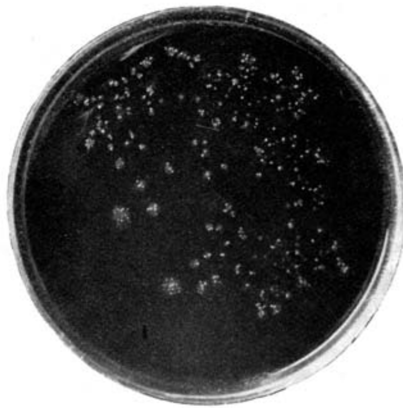

Fig. 1. "B. typhosus." Showing secondary colonies as papillae on isodulcite-agar after five days.

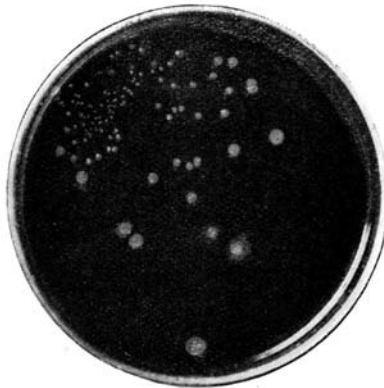

Fig. 2. "B. typhosus." Showing secondary colonies as papillae on dulcite-agar after nine days.

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acid. This second maximum may be maintained for one to eight days and is followed by a gradual or sudden fall.

During the first 12 days the organism is using the dulcite slightly as is evidenced by the fact that the count per c.c. is greater than in peptone water alone during the same time. Daily plating with this strain showed no red colonies during the first week. Reds appeared as a rule after seven and a half days. They remained small in number for a few days until the total count rose suddenly, when simultaneously with this rise the ratio of reds to whites increased.

This late multiplication of organisms is indeed almost entirely due to the production of fermenting individuals as may be seen from the charts.

This course of events suggests some interesting problems. Firstly, what becomes of the whites? Do they multiply and produce reds or not? A number of facts suggest that they do not. In the first place, if whites from a 95 % red plate be put into dulcite broth, they still take the usual time of the untrained strain to ferment dulcite, thus suggesting that they have not been impressed by the training. Secondly, if a typhoid strain be grown on dulcite broth for a few days and then plated on dulcite agar, the papillae do not appear at all on pink colonies, and on the white colonies they do not usually come substantially earlier than they did originally, suggesting that they have not been impressed. This is seen in some of the tables, *e.g.* Table VI.

In the second type of variation the culture in dulcite broth becomes only slightly acid and gives persistently a low total count (see Charts VIII and IX).

One of these never exceeded 126 millions per c.c., and the other never 60 millions. The two of this type both showed two rises in total count, the first starting about the 5th day and the second about the 22nd or 23rd day, the second maximum being less than the first. In each case this later maximum was associated with the appearance of reds on plating on dulcite. The first maximum was also thus associated in one case, but not in the other. After the fall from these maxima the reds disappear to such an extent as to constitute less than 1 % of the total bacterial content, and consequently not to be seen on my plates where I aimed at 80—100 colonies as an ideal number. The persistent low count and the appearance and disappearance of the reds suggest a struggle for existence between the two strains. This varying proportion of reds might partly account for the differences in

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time required to produce acidity when sub-cultures are made at various times.

The liability to contamination in these nine experiments was great, so that towards the end of the time during which these variations were taking place in nine dulcite broths, red and white colonies were selected from the dulcite plates of eight broths and every one agglutinated with a typhoid serum to 1 in 4000, except one white which went up to 1 in 2000. The test-serum had not a very high titre, viz., 1 in 6000, which was the highest with a number of strains, tested by the microscopic method.

The diagrams suggested, in those cases where full acidity was produced, that the counting of the total number per c.c. is sufficient alone to indicate the variation, and this was shown to be, with certain limitations, reliable. If, for example, a typhoid strain trained to ferment dulcite in two days, be counted in dulcite broth on the second day, its count will be about 500 millions per c.c., while a control untrained typhoid will not give more than 200 millions. A one-day dulcite typhoid will give on dulcite broth in 24 hours 800 to 1000 millions per c.c. *B. typhosus* gives 116 millions in 24 hours on saccharose peptone water, in lactose about 126 millions, *i.e.*, not substantially different from the count on peptone water, while a lactose fermenting typhoid gives 470 millions per c.c. in 24 hours, when grown on lactose peptone water.

The capacity to ferment a carbohydrate is apparently of value to the organism. In the case of those strains which had attained the power to ferment dulcite the addition of glucose or dulcite to the peptone water rendered the medium capable of supporting many times the population possible with peptone water alone. The addition of lactose or saccharose, on the other hand, did not lead to increase of the organisms. Conversely the increase of population in the culture on the addition of a carbohydrate may be taken as evidence of the power to attack it by at least some portion of the population. This effect of a carbohydrate may however be missed unless the counts are made both early and over a considerable period. For example, by the addition of glucose to peptone water, an enormous increase of growth occurs in the case of *B. typhosus*, but if one counts on the second day, the living organisms will be fewer in all concentrations of glucose from 0·1 to 4 % than they are in peptone water alone. This is probably due to the high concentration of the acid formed.

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Another source of error is that the training is incomplete and the high total does not show itself till the second or third day. This would be avoided by further training or slightly later counting.

Permanency of the new character.

In respect of the permanency of the dulcite fermenting character I may adduce the following facts: When "Gray" and "Christie" (see Tables I, II and III) had reached the stage of fermenting dulcite broth in one night, red colonies from dulcite plates of these strains were inoculated into peptone water, and sub-cultured 25 times from August 28th, 1909, to January 5th, 1910. It was then found that 100 % of the colonies on dulcite plates were still all intensely red, so that apparently the character had not commenced to recede. Two other one-day dulcite typhoids, carried concurrently with the above to the tenth generation on peptone water, also gave 100 % of reds on dulcite plates.

We have seen that in the training of strain "D" to ferment dulcite the plating showed usually an irregularly increasing ratio of reds to whites if the history in dulcite broth was followed. Now when the ratio was 14 % reds, 90 % reds, and 100 % reds, colonies were picked off to test the permanency of the character at these different stages.

Three red colonies at each stage were inoculated into peptone water and sub-cultured in it ten times between October 29th and December 26th. It was then found that they were all losing their character, *i.e.*, were giving rise to white colonies as well as reds. In the first generation all the progeny were red, in the second, one of the 14 % reds gave only 31 reds out of a total of 45. The other eight of the series gave 100 % of reds. By the fifth generation all the 14 % series and one of the 90 % showed whites, and after the seventh sub-culture all the series showed signs of losing the character.

Variations in individual strains.

It was next determined to further investigate the great differences observed as to degree and time of acidity when *B. typhosus* is grown in dulcite broth. These were thought to depend on the strain used, but this was found to be only a subsidiary factor, as Table VIII shows.

Twenty different colonies from a MacConkey plate of pure typhoid, freshly isolated from faeces, were inoculated into dulcite broth, and the time of acidity noted (see Table VIII).

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TABLE VIII.

21/10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
7th day after seeding	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9th do.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10th do.	—	—	—	?	—	—	—	—	—	—	?	—	—	—	—	—	—	—	—	—
11th do.	—	?	?	,,	—	—	?	—	—	A	,,	?	A¼	—	—	—	—	?	?	?
12th do.	—	A½	A½	A¼	—	—	,,	—	—	—	,,	A¼	A¾	—	—	—	—	,,	,,	,,
13th do.	—	A	,,	A½	—	—	A½	—	—	—	,,	A	A	—	—	—	—	,,	,,	,,
14th do.	—	—	,,	,,	?	—	A	A½	?	—	,,	—	—	?	?	?	—	,,	,,	,,
15th do.	—	—	,,	,,	A½	—	—	,,	,,	—	,,	—	—	,,	,,	,,	—	,,	,,	,,
17th do.	—	—	,,	,,	,,	—	—	,,	A¼	—	,,	—	—	,,	,,	,,	—	,,	A½	,,
18th do.	—	—	,,	A¾	A	—	—	,,	,,	—	A¼	—	—	,,	,,	,,	—	A¼	A¾	A½
19th do.	—	—	,,	,,	—	—	—	,,	,,	—	,,	—	—	,,	,,	,,	—	,,	A	A
21st do.	—	—	,,	,,	—	A½	—	,,	A½	—	,,	—	—	A½	,,	A½	—	,,	—	—
22nd do.	—	—	,,	,,	—	,,	—	,,	,,	—	,,	—	—	,,	,,	,,	—	,,	—	—
24th do.	—	—	,,	,,	—	,,	—	,,	,,	—	,,	—	—	A	,,	A	—	,,	—	—
25th do.	—	—	,,	,,	—	,,	—	,,	,,	—	,,	—	—	—	,,	—	—	A½	—	—
27th do.	A¼	—	,,	,,	—	,,	—	,,	,,	—	—	—	—	—	—	—	—	—	—	—
32nd do.	A½	—	A	A	—	A	—	A	A	—	A½	—	—	—	A½	—	A¼	—	—	—
34th do.	A	—	—	—	—	—	—	—	—	—	A	—	—	—	,,	—	,,	A	—	—
37th do.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A	—	,,	—	—	—

The experiment was repeated with a laboratory strain of *B. typhosus* and 21 tubes were inoculated all from a single colony. The earliest took seven days to produce full acidity and a number never went fully acid, although by the 23rd day acidity appeared in all the tubes. In some, however, it remained slight and indifferent.

In reference to strain, it may be said that if a particular strain usually takes a shorter time than another in producing acidity, it will vary within narrower limits, *e.g.*, strain "Wright" took (see Table I) only five days to produce acidity, and on testing four tubes of it I found they all produced full acidity on the same day, with insignificant variations of degree of colour change. "Christie," "King," "E 10," and "E 8" had three tubes of each tested and showed less uniformity than "Wright," but greater than the strains having a longer usual time (see the following Table IX).

The group of tubes of each strain was inoculated from the same colony. Up to the fifth day they showed no sign of acidity, except Wright.

It was next thought that the size of the seeding might be an important factor. Assuming that the first maximum with *B. typhosus* in dulcite broth is 200 millions per c.c., then if 100 organisms are used

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as a seeding, the number of generations needed to increase this number to 200 millions will be about 20. If, on the other hand, five millions be inoculated, then very few generations, viz., about seven, will produce the same number. Now, other things being equal, the amount of

TABLE IX.

	Christie			King			E 10			E 8			Wright			
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	4
6th day after seeding	A$\frac{1}{4}$	A$\frac{1}{4}$	A$\frac{1}{4}$	A$\frac{1}{4}$	A$\frac{1}{4}$	A$\frac{1}{4}$	A$\frac{1}{4}$	—	A$\frac{1}{4}$	A$\frac{1}{4}$	—	A$\frac{1}{4}$	A$\frac{3}{4}$	A$\frac{3}{4}$	A	A
7th do.	A$\frac{1}{2}$	A$\frac{1}{2}$	A$\frac{1}{2}$	A$\frac{1}{2}$	A$\frac{1}{2}$	A$\frac{1}{2}$	A	A$\frac{1}{2}$	A$\frac{1}{2}$	A$\frac{1}{2}$	—	A$\frac{1}{2}$	A	A	—	—
9th do.	A	,,	,,	A	,,	,,	—	A$\frac{3}{4}$	A$\frac{3}{4}$	,,	—	,,	—	—	—	—
10th do.	—	,,	,,	—	,,	,,	—	,,	,,	,,	—	A	—	—	—	—
13th do.	—	,,	,,	—	A	,,	—	A	A	,,	—	—	—	—	—	—
16th do.	—	,,	,,	—	—	,,	—	—	—	A$\frac{3}{4}$	?	—	—	—	—	—
22nd do.	—	A	A	—	—	A	—	—	—	A	A$\frac{1}{4}$	—	—	—	—	—

selection possible will be proportional to the number of generations, and therefore it seemed reasonable to suppose that the smallest inoculation would cause the earliest acidity. This view led to the following experiment:

Twelve dulcite broths were divided into four groups of three each, and were seeded at the same time from the same culture as follows:

First group with 98 organisms each.

Second group with 19,600 organisms each.

Third group with 1,960,000 organisms each.

Fourth group with 5,880,000 organisms each.

The time of acidity is shown in the following table (Table X).

One of the first group shows a very early acidity. On the other hand the last to get to the stage of "A$\frac{3}{4}$" was a small seeding, so that, speaking in rough general terms, a small seeding will in a series give more irregular results, but not of necessity earlier acidity. If a series is put up with a large seeding, still great irregularity in results is found, because the normal typhoid cells of the same strain differ greatly in their relationship to dulcite. It might be possible with still larger seedings to get uniformity in the time required to produce acidity, but of this I have no positive evidence.

How are we going to class the dulcite fermenting power which *B. typhosus* takes on in this way? Nearly all its strains can be shown capable of producing papillae on dulcite. One-third of them can produce papillae which are red in character on the neutral red medium,

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and in this they correspond with Massini's mutation; on the other hand the new strain in its early stages produces many atavists, *i.e.*, non-fermenting progeny which, apparently, further growth on dulcite removes altogether. This is quite opposed to mutation. Moreover, the fact that long training on dulcite removes the atavistic tendency shows that a new race can be obtained of great stability by long continued selection, a result de Vries could not get with the plants he experimented with.

TABLE X.

	1st Group 98 Organisms as Seeding			2nd Group 19,600 Organisms as Seeding			3rd Group 1,960,000 Organisms as Seeding			4th Group 5,880,000 Organisms as Seeding		
	1	2	3	4	5	6	7	8	9	10	11	12
5th day after seeding	—	—	—	—	—	—	—	—	—	—	—	—
6th do.	—	—	A	—	—	—	—	—	—	—	—	—
12th do.	A½	—	—	—	—	—	—	—	—	—	—	—
13th do.	,,	—	—	A¼	A¼	—	A¼	—	A¼	A¼	—	A¼
15th do.	,,	—	—	,,	,,	—	,,	—	,,	A½	—	,,
16th do.	,,	—	—	,,	,,	A½	A½	—	A½	,,	—	,,
19th do.	,,	A¼	—	A½	A½	,,	,,	A¼	,,	,,	A¼	A½
21st do.	,,	,,	—	,,	A¾	A¾	,,	A½	,,	,,	,,	,,
22nd do.	,,	,,	—	,,	,,	,,	,,	,,	,,	A	A½	,,
23rd do.	A¾	,,	—	,,	,,	,,	,,	A¾	,,	—	A	,,
27th do.	A	,,	—	A¾	,,	A	,,	A	,,	—	—	,,
30th do.	—	,,	—	,,	,,	—	A¾	—	A¾	—	—	,,
32nd do.	—	A½	—	,,	,,	—	,,	—	,,	—	—	A¾
35th do.	—	A¾	—	,,	,,	—	,,	—	,,	—	—	,,

PART II. THE FERMENTATION OF LACTOSE BY THE *B. TYPHOSUS*.

Dr Twort was kind enough to supply me with a typhoid strain which he had trained to ferment lactose, and I examined the same fully, as also its progenitor. Successive sub-cultures on a lactose medium for a period of two years had been required to develop this strain. It showed the following characters: It was an actively motile bacillus and had the biological characters of the normal *B. typhosus* on the ordinary media, except that on broth it produced a marked scum. This latter property will be referred to later.

It fermented glucose, mannite and lactose overnight, without gas formation. Saccharose was not fermented while sorbite was attacked in an irregular fashion. Out of 10 tubes of sorbite only one showed acidity by the ninth day at 37° C. The others showed acidity

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later. This irregularity in the sorbite character belonged to the original normal strain which I had an opportunity of examining, and was in no way due to the lactose variation. The organism did not produce indol.

For the study of its agglutination characters, broth cultures could not be used, as in spite of repeated trial they showed a great tendency to spontaneous agglutination. Agar slopes of 24 hours' growth, however, gave no trouble in this way (*vide* Table XI).

TABLE XI.

Macroscopic Agglutination Tests.

	Dilution	Twort's Lactose Typhoid Strain	G.—A Control Typhoid Strain
Typhoid serum (Wilson)	1 in 20	+	+ + +
	100	+	+ + +
Observations made after 1¼ hrs. and 2½ hrs.	200	+	+ + +
(No change between the two observations)	400	+ +	+ + +
	800	+ + +	+
	1,600	+ + +	−
	3,200	+ + +	+ +
	6,400	+ + +	+
Normal horse serum	1 in 20	+ + +	
	100	+ +	
Observations made after 1¼ hrs. and 2½ hrs.	200	+	
(No change between the two observations)	400	−	
	800	−	
	1,600	−	
	3,200	−	
	6,400	−	
Normal saline solution and typhoid emulsion in 2 hrs.		No agglutination	
Typhoid serum (Wilson)	1 in 20	+ + +	+ + +
Observation after 24 hrs.	100	+ + +	+ + +
	200	+ + +	+ + +
	400	+ + +	+ + +
	800	+ + +	+ + +
	1,600	+ + +	+ +
	3,200	+ + +	+
	6,400	+ + +	+
Normal serum	1 in 20	+ + +	
Observation after 24 hrs.	100	+ + +	
	200	+	
	400	−	
	800	−	
	1,600	−	
	3,200	−	
	6,400	−	

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General result. Twort's trained organism differs only from normal *B. typhosus* by the formation of a scum on broth and by the fact that instead of fermenting sorbite in one day it requires about 10 days, and in some cases does not do it at all definitely in the first culture. The agglutination tests, however, together with all the other fermentation tests except lactose, and also its morphology and motility, show it to be a genuine typhoid bacillus. It is much to be regretted that the organism had these two striking deviations from the type, as it is difficult to say whether they may not have been potent in assisting the training or even of making it possible. Konradi (1904) has stated that *B. typhosus*, after growth for a long period in water, can take on this power of scum formation, so that its presence is not inexorably opposed to the organism being *B. typhosus.* Apart from this statement of Konradi, however, I can find no reference in the literature to the formation of a scum on nutrient broth by the *B. typhosus.*

To ascertain whether the organism was a pure strain in reference to the lactose-fermenting character, it was plated out on MacConkey's medium, *i.e.* neutral red lactose bile salt agar, and incubated at 37° C. On the second day after plating a mixture of red, salmon-pink and white colonies was present. Up to the sixth day the salmon-pink colonies tended to become a little deeper in colour but subsequently showed no further change. The difference in colour is not entirely a question of crowding as some of the white colonies were standing alone in similar positions to red colonies. This was repeated on two plates when the reds amounted to 50 per cent. of the total, the non-crowded portions only being counted.

The plating was also undertaken on lactose agar without bile salt. The only difference that could be detected was that the percentage of reds in the earlier days was less than on MacConkey's medium. The absolute number of reds continued to increase until the twelfth day.

The plating was also undertaken from lactose-peptone-water a few days old and fully acid, when it was found that the percentage of reds on the MacConkey plates on the sixth day reached 95 per cent.

Plates were also made on the same medium from the scum on the broth, and it was found that the reds were fewer and later in appearing than those taken from the broth itself. The difference was, however, not very marked.

As the above facts pointed to great instability in respect of lactose-fermenting power, five red colonies and five white were picked off and put on to agar slopes for further examination.

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The lactose-fermenting and non-fermenting strains, which for the sake of brevity I shall call the red and white respectively, agreed in all their characters, except in the power of fermenting lactose. All the five red strains gave acidity in lactose-peptone-water overnight, while only two of the white strains became acid, although they were observed for a month, viz., one on the seventh and one on the ninth day. No indol was produced by any of the strains. Agglutination and absorption tests gave the following result:

TABLE XII.

Typhoid Serum (Wilson, Titre 1 in 12,000). Macroscopic Method.

Typhoid serum	Red strain after 2 hours	White strain after 2 hours
1 in 50	+	–
100	+ +	+
200	+ + +	+ +
400	+ + +	+ + +
800	+ + +	+ + +
1,600	+ + +	+ + +
3,200	+ + +	+ + +
6,400	+ + +	+ + +
Normal horse serum		
1 in 50	+	+ +
100	–	+
200	–	–

The above typhoid serum was absorbed with the white strain by Castellani's method, and it was found to absorb all the agglutinin for itself, the red strain, and also a stock typhoid strain, in dilutions from 1 in 150 to 1 in 5,000 (Table XIII).

TABLE XIII.

Typhoid Serum (Wilson) absorbed with white strain. Macroscopic Method. Observed after 2 hours.

Dil. of Serum after being absorbed	White strain	Red strain	Strain L
1 in 50	+	+ +	+
150	–	–	–
500	–	–	–
1,000	–	–	–
5,000	–	–	–

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A partial absorption by "H," another known strain of typhoid, gave the following results with the same serum (Table XIV).

TABLE XIV.

Typhoid Serum absorbed with "H."

Serum	"H"	"G"	Red Twort
1 in 100	+ + +	As "H"	+ + +
200	+ + +	,,	+ + +
400	+ + +	,,	+ +
800	+ +	,,	+
1,600	+	,,	–
3,200	–	,,	–

The titre of the serum with "H" was 1 in 12,800.

Another partial absorption of the same serum with "H" gave maximum agglutination with "H" and the red strain organisms at 1 in 100.

That these strains, therefore, both red and white, were identical except in respect of the lactose-character was manifest. So far as agglutination tests went, they were both genuine typhoid organisms.

Investigation of the progeny of the red and white colonies.

Red, salmon-pink and white colonies were plated out from broth on MacConkey's medium in order to study the percentage of the particular colonies in the progeny. Botanists find that the selection of races is best furthered, not by selecting divergent individuals, but rather by selecting individuals, which in their progeny show an average high development of the divergent character. This is expressed by the percentage which conforms to some arbitrary standard, the "hereditary percentage." I attempted the same method here and found that like colonies gave very different results on plating. The results stated are obtained by taking two separate sets together.

The red strains gave on the third day 96 % of reds and this percentage did not increase. In none of the colonies was the redness so intense as when the plating took place from lactose-peptone-water a few days old.

The salmon-pink colonies on plating gave a mixture of reds and pinks on the one hand and whites on the other. On the fifth day the fermenting colonies were in excess.

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The white colonies on plating gave only whites though observed for fourteen days. Each of the above observations was made from a consideration of two sets of plates.

The position would appear to be the following: the red strain is constantly throwing off atavists of white and red; the salmon-pink produces red, salmon and white; but the whites show no tendency to produce progeny of the new variety.

Rapid sub-culture of the red strain on peptone water without lactose was next undertaken, the sub-cultures being made every three days, in order to see if the lactose-fermenting property would be rapidly lost. Two series of cultures were so treated, derived from different red colonies. The fifth sub-culture plated out on lactose without the bile salt showed after four days, no red colonies in either set of plates. The same sub-cultures on MacConkey's medium showed no red colonies in one set of plates, but on the set from the second culture 6 % of red colonies appeared.

The tenth sub-culture was plated out on MacConkey's medium and on the fourth day no red colonies were present in either set. On the fifth day, however, a few salmon-pink colonies appeared in the crowded portion of one plate, but none of the large independent colonies showed the slightest suspicion of acidity. The pink central colonies took three days to produce acidity in lactose-peptone-water. Some whites tested produced no acidity in ten days.

The two series of red strains were plated out again on MacConkey's medium after the twentieth sub-culture on peptone water. They were observed daily for 12 days and during that time did not show a single red or pink colony, either in the crowded or scattered portions of the plates, though some of the colonies were half an inch in diameter by the fifth day.

The sixth sub-culture on peptone water was inoculated into litmus-lactose broth (two tubes) and produced acidity, one on the fourth day and the other on the fifth day. The fifteenth sub-cultures were inoculated into four tubes of litmus-lactose-peptone water. One became acid on the seventh day, two on the eighth day, and one on the eleventh.

It is evident, therefore, that though the organism was capable of fermenting lactose it was not in a position of stable equilibrium and was always reverting towards its normal condition.

It seems permissible to say that the greater the number of generations passed through in peptone water, *i.e.*, the longer the period since

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the selective process has been in abeyance, the nearer will the average of the progeny approach the normal.

This lactose-fermenting typhoid can scarcely be dignified with the title of a new variety on account of this constant tendency to reversion. This same tendency was present in the early stage of the dulcite training, however, but, as we have seen, a prolongation of the training produced a stable and pure race. The stable dulcite position can be sometimes attained in two to three months' training, while two years' training in the case of lactose is required to bring it to the early stage, *i.e.*, of producing fermenting progeny always tending to throw off atavists. The early stage in dulcite training is attained in two to ten days, and if the analogy could be permissibly carried further, it seems only natural to believe that a stable and perfectly constant lactose-fermenting race of typhoid, proving itself to be such after 20 or 30 sub-cultures on peptone water, would require very many years of this continuous training-process for its selection.

This process might be quickened by the systematic use of colony selection as the sub-cultures were made.

Author's attempts to produce a lactose-fermenting strain of the "B. typhosus."

The training of *B. typhosus* to ferment lactose was next undertaken. For this purpose, many normal strains were used and also the white atavists of Twort's lactose-fermenting typhoid strain which we have just been studying.

The training was carried out on lactose agar slopes with neutral-red and also on litmus-lactose-peptone water. The solid medium was necessary in order to ascertain if papillae were formed as in the case of mutation.

Table XV gives the results of training *B. typhosus* on neutral red lactose agar in order to select out a lactose-fermenting strain. White atavists of Twort's strain were used and also other ordinary strains of *B. typhosus*.

The three separate strains of white atavists of the lactose-fermenting typhoid strain during retraining on a solid medium containing lactose, produced papillae, small at first, becoming on further sub-culture large and red, and followed on still further sub-culture by small papillae which ultimately coalesced to form a broad thick deep coli-red streak. All the three strains agreed in the formation of large red papillae, but the

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TABLE XV.

To show the history of training various typhoid strains on neutral-red-lactose-agar slopes.

Organisms and results of examination.

Dates of sub-culture	White Atavists of Twort's Lactose typhoid (1)	(2)	(3)	G. A Laboratory Strain of Normal *B. typhosus* (1)	(2)	(3)	L. A Second Strain of Normal *B. typhosus* (1)	(2)	(3)
5th March, 1909	—	—	—	—	—	—	—	—	—
15th ,, ,,	—	—	—	—	—	—	—	—	—
15th April ,,	—	—	—	—	—	—	—	—	—
3rd May ,,	Shows fairly large papillae of neutral reaction	*Large acid papillae develop*	Smooth growth	Developed tiny neutral papillae	—	—	—	—	—
24th May ,,	*Large acid papillae develop*	A thick dirty reddish streak appears without papillae	,,	,,	Tiny papillae of neutral reaction developed	—	—	—	—
22nd June ,,	Small papillae develop of similar colour to rest of growth	Small papillae appear	,,	—	—	—	—	—	—
18th August ,,	,,	,,	Small neutral papillae appear by the fifth day	—	Developed tiny papillae of neutral reaction	—	—	—	—
20th Sept. ,,	Growth remained smooth, the acid reaction fading slowly	Small papillae appeared by the eleventh day	*Large coarse papillae of acid reaction*	Developed tiny neutral papillae	—	—	—	—	—
10th October ,,	,,	Growth remained smooth	Growth remained smooth	—	—	—	—	—	—
11th Nov. ,,	,,	,,	,,	—	Developed tiny papillae	—	—	—	—
10th Dec. ,,	,,	,,	,,	Developed tiny neutral papillae	—	Developed tiny neutral papillae	Small papillae appear	Small papillae appear	Small papillae appear

The cultures which are not marked in showed during their whole growth no papillae formation.

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time of formation was not always the same, though the training ran concurrently in all. This shows that there is considerable variety amongst the atavists.

The training of normal typhoid strains of the same series shows in the first year a tendency to the appearance of small papillae in from 6 to 20 days after the sub-culture is made. These may appear repeatedly on the same strain; they have the same colour as the rest of the growth, and are very small, quite unlike the large papillae observed in the later stages of training of the before-mentioned lactose-atavists.

The significance of these papillae is difficult to determine. They may be due to the lactose in which a selection occurs, in favour of organisms using it rather better than their neighbours though unable to produce an acid reaction in the medium, but it is also possible that they may indicate a selection going on in favour of strains which can use the peptone basis of the medium more fully. I incline to the former view. It seems the explanation of the large papillae with acid reaction, as they are accompanied by such an augmentation in their fermenting power on lactose. In the case of the small papillae which frequently recur, I believe them to be also due to the lactose, since papillae on nutrient agar without sugar are not frequent and the papillae have a somewhat different appearance. On nutrient agar they are slightly larger and not so sharply defined from the growth on which they occur.

The three white atavists of Twort's lactose-typhoid strain that did not ferment lactose were sub-cultured into litmus-lactose-peptone-water after one month, and then again after a second month. On the sixth day after this sub-culture, white strain I became fully acid. On the eighteenth day after sub-culture, strain II showed distinct but not complete acidity, while the third remained unaffected until the twentieth day, when it showed slight acidity. The controls of ordinary *B. typhosus*, three of "G" and three of "L," were unaffected. Sub-culture was again undertaken after one month and all showed slight acidity two days after the sub-culture. On the third day they were all fully acid. Sub-culture was again undertaken after a month, and on the second day after, one strain was fully acid, the other two half-way turned. The following day they were all fully acid. Sub-cultured again after an interval of seven weeks, one became acid on the first day. The last sub-culture was made at a long interval, from unavoidable causes. At a time when the fourth sub-cultures showed acidity in all the tubes, viz., on the fifth day, it was sub-cultured to see if it was necessary to

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have these long intervals between the sub-cultures. They all showed slight acidity on the second day again, but no sign of it on the first. The fourth sub-cultures plated out on MacConkey's medium, five days after their inoculation, showed no sign of acidity until the second day, when white strain I showed a few pale salmon tinted colonies, the other two strains, white only. On the fourth day, I and II both showed salmon colonies; III showed a very few red ones. They showed no subsequent change.

Three strains of "G" and three of "L" have been growing on litmus-lactose-peptone-water since February (1909), being sub-cultured each month. At the end of a fortnight they only give an indefinite acid reaction. Compared with a control they apparently show slight acidity, but it is not definite. I have also trained Twort's normal typhoid strain from which he developed his lactose fermenter, since March 31, 1909, up to date (November, 1910), by means of fortnightly sub-culture in the case of three series, and monthly sub-culture in another three series, and while they both at the end of a fortnight appear identical and very slightly acid, the training appears to have been so far of but little value.

On June 3, 1909, I put a further series of 12 different typhoid strains on lactose-peptone-water, some fresh from the body and some old. They have been sub-cultured every month up to date. They are all difficult to train as far as one can at present judge. I also attempted the training during four months (January—April, 1910) of various dulcite-fermenting typhoids, but with no greater success. So far as I can judge, therefore, normal typhoid strains cannot acquire this character by this method of training in one year. Atavists of a trained lactose-fermenting typhoid can be fairly easily retrained.

The training of typhoid to ferment lactose was also undertaken by more rapid sub-culture, viz., every four or five days, and this was continued in the case of "G" (two strains) for 42 generations, and in two strains of another typhoid organism for 38 generations, without any more effect than was produced in the slow series of sub-cultures carried on during the same time. Plating out on MacConkey's medium at any stage of the above training failed to produce red colonies.

A series after five monthly sub-cultures on lactose-peptone-water was then sub-cultured every week for 13 generations and produced nothing more than slight indefinite late acidity.

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Part III. Typhoid and Isodulcite.

Reiner Müller has described the formation of papillae on isodulcite by the *B. typhosus* and found that they might be composed either of fermenters or non-fermenters of isodulcite, that is to say, they might be red or not, on media with a suitable indicator. I have tested 20 strains of *B. typhosus* from different sources on neutral-red-iso-dulcite-agar and have found a well-defined papillae-formation appear with every strain, in from three to five days. The papillae might reach 30 or even more in number and were present in all the large discrete colonies. In no case, however, did I find the papillae red. All these strains were inoculated into ordinary broth from a single papilla and replated on isodulcite-neutral-red-agar with the result that I obtained in nearly every case two sorts of colonies, the first showing quite large naked-eye papillae, the second smooth to the naked eye and of denser growth, but in no single instance red in colour. The examination of these smooth colonies with a lens frequently reveals very tiny papillae, but not at all similar to the original ones.

Dulcite- and lactose-trained typhoids give these papillae on iso-dulcite.

As Reiner Müller occasionally obtained acid papillae, which when sub-cultured gave permanently acid strains, I observed the time taken by a few strains to produce acidity in isodulcite broth. The lactose-typhoid took 19 days, but when sub-cultured after 21 days it became fully acid in 11 days and a second sub-culture after similar treatment produced full acidity in two days.

A dulcite-fermenting typhoid strain required in the first isodulcite-broth culture 19 days, on first sub-culture six days, and on the second two days.

A normal typhoid strain "D" took only seven days in the first broth-culture, four in the second, and two in the third.

Another stock typhoid strain was very obstinate, and after the third monthly sub-culture it took 21 days to produce full acidity; after the fifth sub-culture it became fully acid in three days.

Two other strains of typhoid occupied an intermediate position in respect of time taken to acquire the fermenting power on this particular carbohydrate.

The testing of the constancy of this character (*i.e.* absence of papillae-forming power) to obtain evidence of mutation is only of value in the

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presence of a positive result. To carry out the test a number (four) of typhoid strains varied towards isodulcite so that they grew on it luxuriantly without being able to produce papillae, were put through 13 generations of peptone water during three months and on replating on isodulcite not one of them produced papillae again. If in this case the evidence of bacterial mutation had not been positive it would have been difficult to draw any conclusion, as the *B. typhosus* occasionally produces papillae on ordinary nutrient agar for some cause as yet undefined.

Subvariants. The first indication of the acquirement of the dulcite character may be the appearance of salmon-pink colonies, the centres not being markedly deeper in colour than the rest of the colony, and this probably must be looked upon as a subvariant. I have sometimes found dulcite broth almost entirely composed of these elements. More frequently, however, the new character is shown by a small deeply coloured spot in the centre of an otherwise white colony. In this connection I might mention that plating out from broths in which mutation is occurring, at frequent short intervals, *e.g.* every hour, and observing the character of the cells, does not appear to have been done, so that the assumption that bacterial mutation exists (without the occurrence of subvariants in the process) as in botanical mutation is not yet absolutely proved.

SUMMARY OF RESULTS.

(1) *B. typhosus* ferments *dulcite*-peptone-water in about 10 days. By sub-culture this is readily shortened to one day. It produces non-fermenting colonies on dulcite-agar plates. The colonies show after five days and upwards, secondary colonies as papillae, which may or may not have an acid reaction.

Growth of *B. typhosus* on dulcite media readily gives rise to bacilli forming, from the commencement, acid colonies on dulcite plates. The dulcite character has a tendency to atavism in the early stage which further growth on dulcite removes.

(2) *B. typhosus* can be trained to ferment lactose, only with very great difficulty. Definite and conclusive results have been obtained by me only with the white atavists of Twort's trained lactose strain. Other strains (20) have not yielded to such training methods, although many of them have been trained for over a year. The lactose character

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when acquired is very unstable, and is soon lost when growth takes place in a lactose-free medium. A fermenting colony on replating gives rise to a mixture of fermenting and non-fermenting colonies.

(3) All strains of *B. typhosus* which I have examined show the secondary colonies on isodulcite described by Reiner Müller. Growth on isodulcite yields directly a strain without this papillae-forming power. So-called "acid" papillae as described by Reiner Müller have not been observed in the strains (20) investigated by me. This new strain does not regain the power to form isodulcite papillae by frequent sub-culture apart from isodulcite. Apparently the acquired character has arisen by mutation. Fermentation of isodulcite requires a considerably longer process of training.

(4) The above three characters vary in permanency. The permanency varies inversely as the time required for the selection or training.

(5) Papillae-formation arising on carbohydrate media on colonies of members of the typhoid-coli group may indicate variation by slow degrees just as well as by mutation. I have observed it in normal typhoid varying towards dulcite and lactose as well as isodulcite. In the case of Twort's strain which fermented sorbite slowly, I observed papillae-formation on sorbite-agar.

Formation of papillae simply indicates apparently that the variation only affects very few individuals of the colony to a great extent, and does not guarantee permanency in the character.

If the papillae arise early and without sub-culture, the evidence adduced so far suggests that the character will have considerable permanency. In the case of dulcite the results show that the formation of papillae on certain carbohydrate media cannot, as Reiner Müller stated, be regarded as definitely specific for the variety of micro-organism exhibiting them.

The variations detailed in the above communication do not in our view invalidate the sugar tests as aids to differentiation, since these chemical functions are subject to variation probably to no greater extent than other functions of bacteria. Further, the ability of an organism to vary in any particular direction may be of considerable value in differential diagnosis.

This instability makes it imperative to have as large a number of differentiating characters as possible for every species, and also to take care in laboratory culture that we do not grow bacteria under conditions likely to alter their characters. It would be inadvisable for example

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to propagate the *B. typhosus* from a dulcite culture a few days old if one desired it to conform to the normal typhoid type in respect of dulcite.

In sending this paper to the Press I would like to acknowledge my indebtedness to Dr Martin, who has supervised my work on behalf of the British Medical Association, and to Dr Ledingham, who suggested the work, from both of whom I have received much valuable help and criticism. Many other colleagues at the Lister Institute have helped me from time to time in points of difficulty, and I take this opportunity of thanking them.

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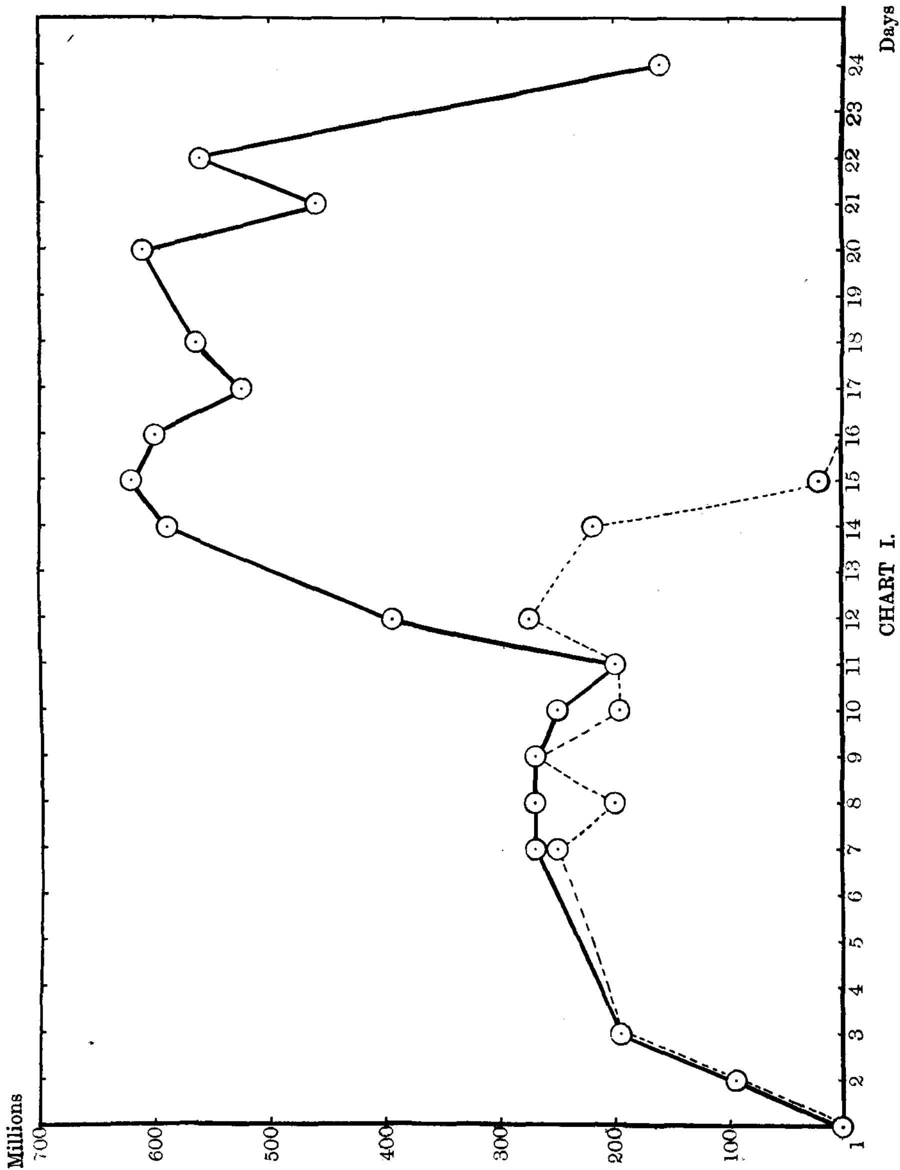

CHART I.

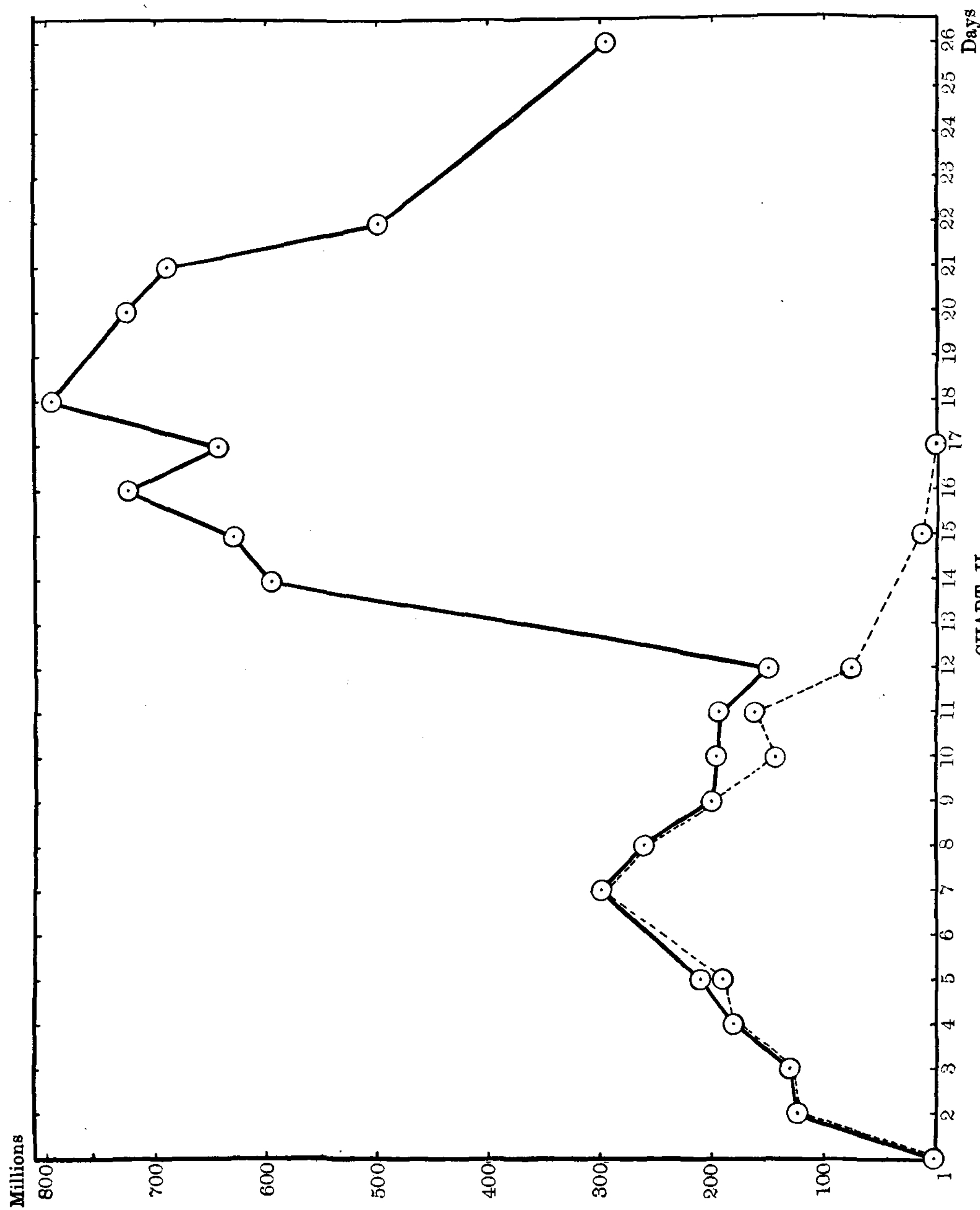

CHART II.

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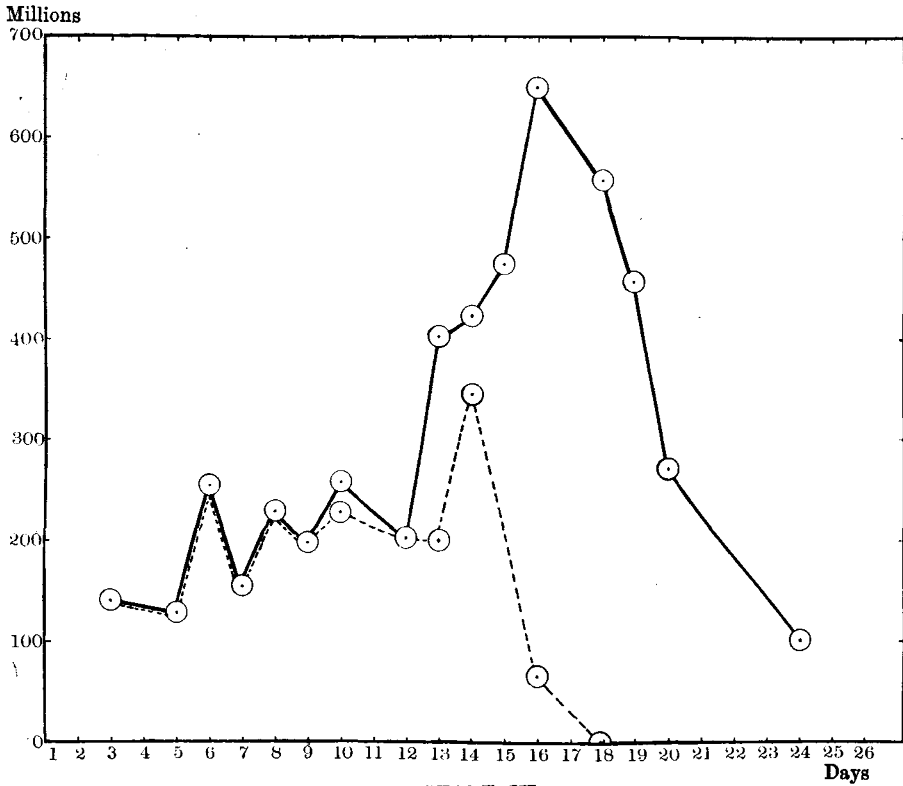

CHART III.

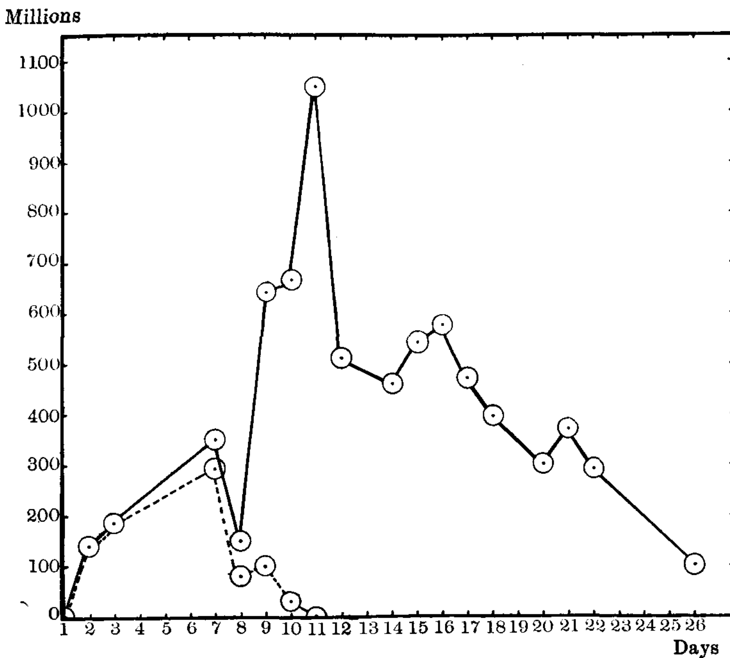

CHART IV.

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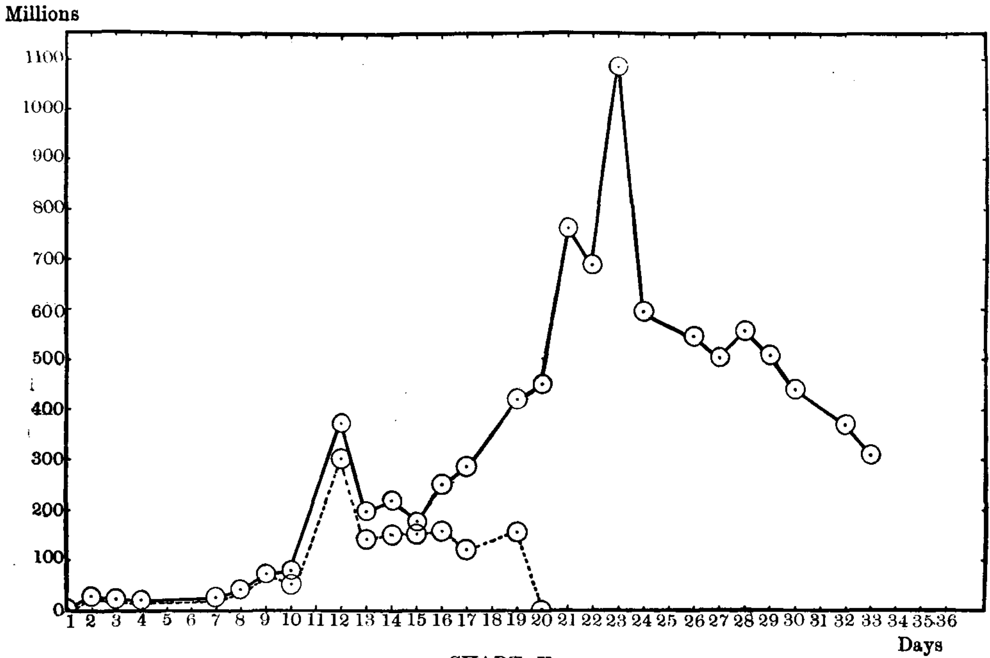

CHART V.

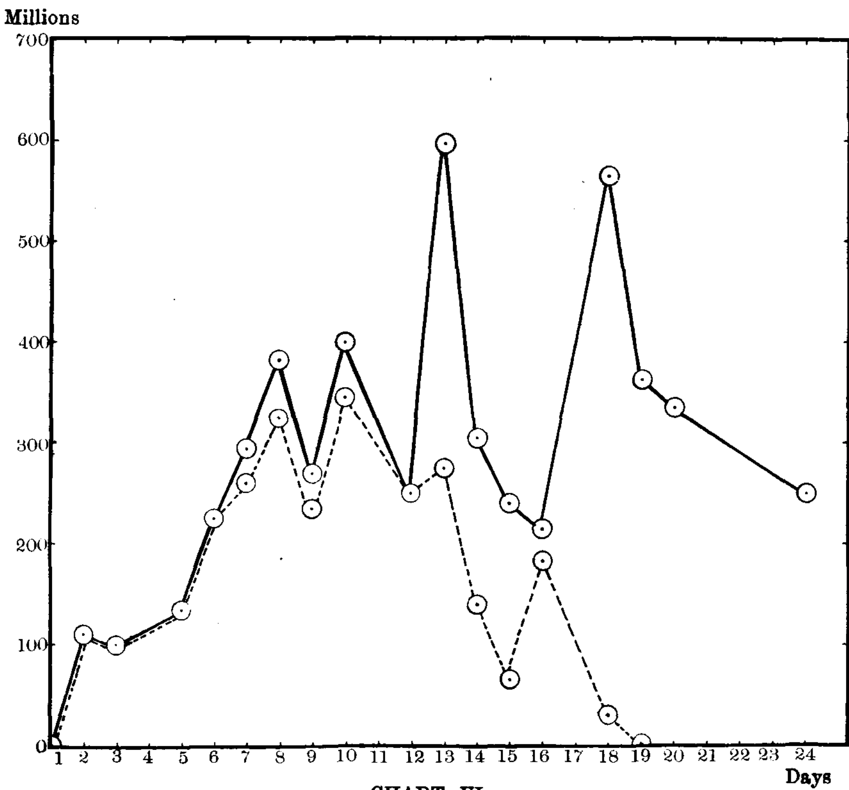

CHART VI.

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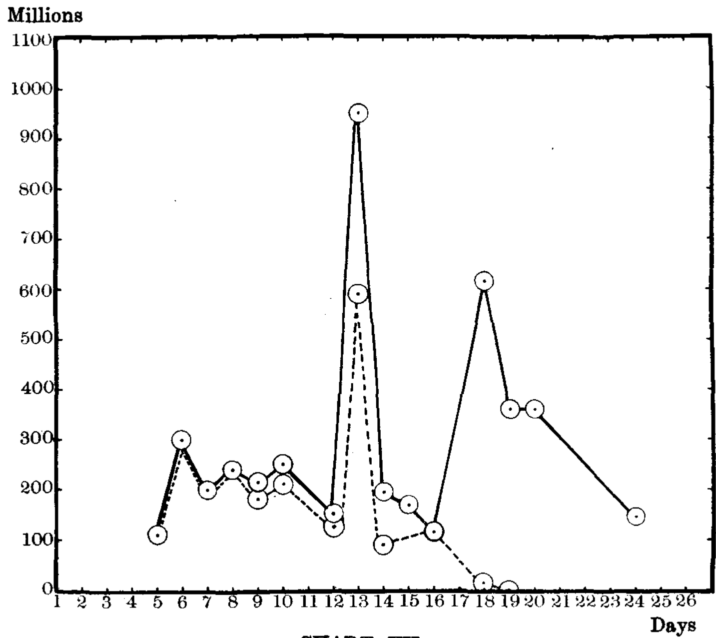

CHART VII.

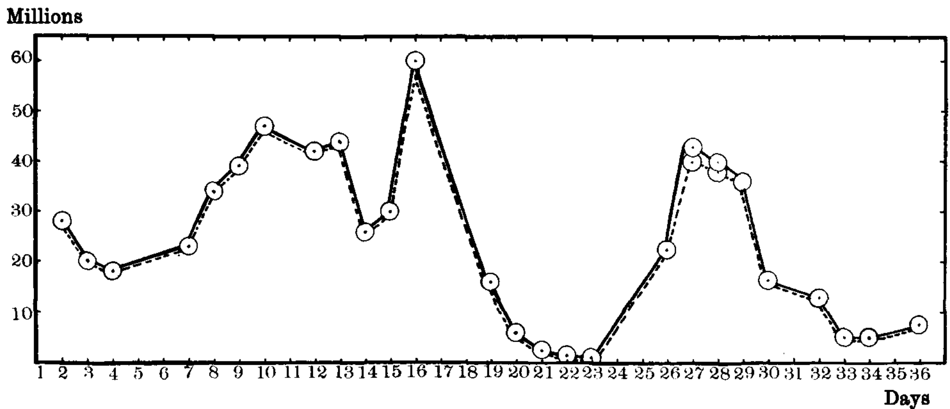

CHART VIII.

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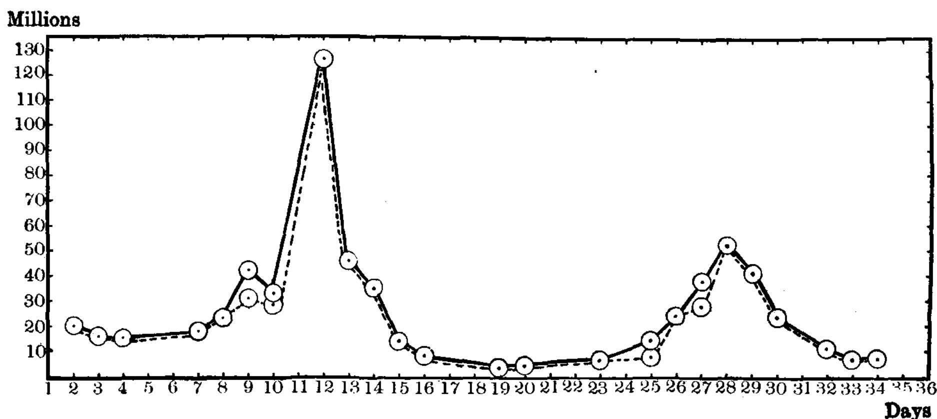

CHART IX.

EXPLANATION OF CHARTS.

Charts I to VII. The upper line shows the total count in dulcite broth from day to day during the process of variation expressed as millions per c.c.

The dotted line indicates the numbers of non-fermenting elements in the broth obtained by considering the total count and the ratio of red to white colonies on neutral-red-dulcite-agar plates.

This gives the history of a dulcite broth becoming fully acid in about a fortnight.

Chart V shows that the dulcite broth had a very low total count per c.c. for ten days and thus appeared to be of the second type. At this stage, however, it changed suddenly and became like the first type almost in all respects.

Charts VI and VII show a sudden increase in numbers; coincident with this increase the acidity rises and the subsequent rapid death is presumably due to this cause. A few days later a similar sudden increase and diminution occurs. Had this happened only in one culture, it might have been due to clumping, but the identity of the two charts makes me believe such an explanation is insufficient.

It seems more likely that the first selection, under the influence of which a dulcite-fermenting strain was produced, led inevitably to increased acidity which occasioned the death of the majority of the individuals, leaving only those more resistant to this adverse influence. Subsequently a new stock was bred from these, to be in their turn destroyed by a further rise in acidity.

I have found for example that *B. coli* (Escherich) survives ten days' growth on lactose-peptone water at 37° C. and *B. coli* (Durham) 13 days. But a recently selected *B. coli* like the lactose-fermenting strain of *B. coli mutabile* (Weiss) cannot be sub-cultured after two days' growth on lactose-peptone water at 37° C. The recently selected lactose-fermenting strain is evidently much less resistant to the acid produced than the natural varieties of *B. coli* which ferment lactose without previous selection.

Charts IV and V show also sudden falls in the total count.

Charts VIII and IX. To show the march of events in the type which does not attain full acidity in the dulcite broth. Observe that the non-fermenting elements predominate throughout, though on two occasions with a rising total count, reds appeared on the plates in the case of (*a*), but only during the second maximum in case of (*b*).

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