VARIATIONS IN TYPHOID BACILLI¹

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INTRODUCTION

Just as there is often great difficulty in diagnosing atypical clinical conditions, so great difficulty may be experienced in identifying bacteria which develop abnormal characteristics. The acquisition, by individual strains of many species of bacteria, of morphological and cultural characteristics which differ from the usual type has been noted by many observers and has been referred to more or less loosely by several different terms. Thus, Neisser (1906) and Massini (1907) used the word "mutation" to designate atypical forms of *Bact. coli*, Pringsheim (1911) speaks of an "adaptation" of bacteria, and Gurney-Dixon (1919) uses the term "transmutation."

We can sometimes follow such variations by gradual changes, from one stage to another, during which the bacteria pass through a process of evolution, adapting themselves to their surroundings.

Such variations may consist in the acquisition of new morphological, biochemical, or serological characters, in the loss of similar properties, or the two processes may occur at one and the same time. The change may be sudden or gradual, and is generally retained by the offspring.

The study of such variations is of fundamental importance to an understanding of the bacteria and may have considerable botanical importance since it would seem that processes of evolution or adaptation could be most easily investigated with

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forms whose characteristics are easily studied and in which generation follows generation with such speed that observation over the period of a year or longer might correspond to ages of development among the higher species. Moreover, from a purely practical point of view it is necessary to know to some extent just how much and how permanent a degree of variation is to be expected when well-known species are subjected to alternation between the conditions prevalent in artificial media and those existing in the human and animal bodies.

In the following studies the writer has occupied himself exclusively with the changes observed in the typhoid bacillus.

Strains

The cultures employed in this study were 138 in number and had been carried on artificial mediums since their isolation from patients. They were divided into three groups, as follows:

1. The stock cultures of the United States Army Medical School . . 116
2. Cultures collected by Dr. Oscar Teague. 10
3. Cultures collected by Lieut. R. C. Colwell in France, and given
us by courtesy of Lieutenant-Colonel Nichols 12

The sources of these cultures were as follows:

	<i>strains</i>
Blood cultures	55
Stool cultures	15
Urine cultures	11
Bile cultures	1
Sources unknown	56

Duration of cultivation on artificial media:

Over two years	15
One to two years	25
Six to twelve months	42
Less than six months	32
Recent isolation	11
Age unknown	13

Media and technique

In our experiments the media used and technique employed were as follows:

(a) Meat infusion broth. Meat infusion inoculated with *Bact. coli*, incubated about twenty-four hours at 37°C., autoclaved and filtered. To this was added 1 per cent pepton and 0.5 per cent sodium chloride.

(b) Nutrose broth. Instead of meat infusion 0.25 per cent nutrose was used. Both broth media were autoclaved for fifteen minutes at 15 pounds' pressure, and the reaction was adjusted to pH 7.0 or pH 7.1.

(c) Media containing sugars. In order to diminish the risk of decomposing the sugars during sterilization, they were dissolved in sterile distilled water, and heated in the autoclave for ten minutes at 10 pounds' pressure.

The sterilized sugar solution was added in the proportion of 1 per cent to the sterile broth together with 5 cc. of sterilized litmus or 5 cc. of 2 per cent phenol red and 1.2 cc. of decolorized 1 per cent aqueous solution of china blue (Morishima, 1917) per 100 cc. of the broth. Then the medium was transferred to small test tubes, and allowed to stand at least twenty-four hours at 37°C. in an incubator and for twenty-four hours at room temperature before being used.

For plates meat infusion agar (2 per cent) containing 1 per cent of the sugar was used. Decolorized china blue was then added in the proportion given above for the fluid medium. The reaction of all media mentioned above was adjusted to pH = 7.0 or pH = 7.1 by means of phenol red.

The stock cultures were transferred to agar slopes and incubated over night. Then pepton water tubes (1 per cent pepton 0.5 per cent salt solution, reaction pH 7.0) were inoculated from the slant cultures. After the latter had been incubated over night, one loopful of the pepton water growth was transferred to each tube of sugar medium; agglutination tests with the pepton cultures were also carried out.

In plating cultures on Endo or any other plates, one or two loopfuls of bacterial suspensions were usually streaked close to the margin of the Petri dish. The plate was divided into five parts by lines drawn on its bottom. From the first streak made with the loop, the suspension was spread over one-fifth of the surface; from the border of this area over the next third, and then from the last border over the remaining surface. By this method the distribution of bacteria was found to be satisfactory (Morishima, 1917). They were then kept in the incuba-

tor until the end of the experiment. Endo plates were occasionally inoculated from the sugar media in order to control possible contamination of the latter.

I. VARIATIONS IN THE UTILIZATION OF CARBOHYDRATES

Variations in the biological behavior of the typhoid bacillus have been the subject of a great deal of investigation but, in the earlier work, especially, the identification of the races under observation was often incomplete (at least as far as one can judge from the publications) and all the reported results cannot be accepted without analysis.

Some of the earliest work was done on indol formation and on the fermentation of lactose.

Miss Peckham (1897) induced indol formation in a number of strains of *Bact. typhosum*.

Wilson (1902) isolated a strain from a typhoid carrier which produced acidity in lactose media at 22°C. while it did not produce it at 37°C. and the strain agglutinated only in 1:50 dilution of a typhoid serum of high titre. In other respects it resembled typical typhoid strains.

Klotz (1904) isolated from the St. Lawrence River water, a typhoid-like organism which he called *B. perturbans*. It fermented lactose and sucrose, formed indol, produced acidity in milk without coagulation and agglutinated with 1:2480 dilution of anti-typhoid serum.

McNaught (1905) isolated two organisms which he named *B. typhosus similans*, one of them from harbor water, the other from a well. The former did not produce indol and the latter did. Neither agglutinated in anti-typhoid serum. Both were motile when isolated but after some days of cultivation lost their motility.

Klotz's strain isolated from the water of the St. Lawrence River; McNaught's *B. typhosus similans*; and Wilson's strain isolated from a typhoid carrier's stool cannot be definitely accepted as real typhoid bacilli, because they were not sufficiently investigated to determine this fact positively.

Mandelbaum (1912) obtained a bacillus from the blood or feces of more than fifty patients with clinical typhoid fever in Munich, which he named *B. metatyphi*. This bacillus resembled *Bact. typhosum* in all respects except that it produced alkali instead of acid in media con-

taining glycerol. He showed that these cases were infected, in all probability from the same typhoid carrier, a woman who served as a milker in a dairy near Munich. This woman harbored both typical and atypical bacilli. The *B. metatyphi* retained the property of producing alkali in glycerol medium for five and one-half years when transplanted on plain nutrient agar. Russowici (1908) reported one case of *B. metatyphi*, and Ditthorn and Luerssen (1912) reported two similar cases.

Jacobsen obtained (1910) a bacillus which he described as *B. typhi mutabile* from a small epidemic of clinical typhoid fever in an insane asylum in Denmark. It resembled *Bact. typhosum* in all respects except the following:

1. It fermented mannitol after fifty hours.
2. Its growth was strongly inhibited on Conradi-Drigalski agar or plain agar which had been autoclaved.
3. Cultures from the plates showing retarded growth did not agglutinate in typhoid immune serum, but cultures of the same strain on media yielding good growth gave typical agglutination with typhoid immune serum and resembled *Bact. typhosum* in all other respects. *B. typhi mutabile* gave good specific agglutination five months after its isolation. There was normal growth on the Endo plates which removed the inhibitory action exerted on this strain by other media.

Fromme (1911) reports a bacillus, the growth of which was retarded on nutrient agar but which grew in ascitic fluid, human blood, guinea pig's blood, rabbit's blood and egg-yolk or on agar to which sodium sulphite had been added. His bacillus differed from Jacobsen's in that it agglutinated with typhoid immune serum from the start.

The variants of typhoid bacilli—*B. metatyphid* (Mandelbaum), *B. typhi mutabile* (Jacobsen) and the xylose non-fermenter of Weiss are unquestionably true *Bact. typhosum*.

Twort (1907) after growing a strain of *Bact. typhosum* for two years in lactose media succeeded in producing a strain that fermented lactose. He also conducted special experiments with a typhoid bacillus which had acquired the power of fermenting dulcitol. When such a culture was plated out on agar, subcultures from single colonies retained the dulcitol splitting powers, although they were still capable of being agglutinated by a typhoid immune serum, thus proving that the fermentation was not due to any contaminating microbe. On inoculating the dulcitol-fermenting typhoid culture into a guinea pig, subcultures were obtained showing the same reactions and these reactions were also maintained, even when the organism was grown for several

generations on ordinary pepton agar. His conclusions were that the sugar fermenting powers of an organism may be artificially changed by growing the said organism for a succession of generations in media containing a sugar which at the commencement of the experiment it was unable to ferment.

Kuwabara (1907, 1909) under the direction of Dr. Shiga isolated an atypical typhoid in addition to a typical one from a typhoid patient's stool. It fermented lactose, sucrose, and milk-whey as *Bact. coli* does and produced reddish color on Endo and Conradi-Drigalski plates but agglutinated in antityphoid rabbit serum in high dilutions just as a normal culture of typhoid bacilli did. After twelve to fifteen passages through plain laboratory nutrient media these atypical characters all disappeared.

The results of Twort and Kuwabara amount practically to a complete alteration of the identification characteristics of the typhoid bacillus. These observations are of the greatest theoretical importance but fortunately strains of this nature have been produced or observed so rarely that they cannot be regarded as a practically important factor of confusion in identification. This is apparent from Penfold's work cited below.

Penfold (1910a, 1910b, 1911, 1914) as cited by Dixon, working with twenty strains and carrying many of them for more than a year in a lactose medium, obtained only negative results; he showed that the Twort lactose fermenting strain gave rise to daughter colonies on lactose agar. This Twort culture fermented sorbitol in broth only after a number of days and Penfold found that it also gave rise to daughter colonies on sorbitol agar. He observed with some of his cultures late acid production in rhamnose broth and on transplanting from rhamnose broth to rhamnose broth after several weeks of incubation, he was able to obtain subcultures which fermented in one, two or three days. He also made a very careful study of the behavior of *Bact. typhosum* in dulcitol broth and on neutral red dulcitol agar. In one of his experiments in which fourteen strains were inoculated into dulcitol broth the first signs of acidity occurred in from five to fifteen days. If, after one month, subcultures were made in new dulcitol broth, an acid reaction was produced in from one to four days. Subcultures, which had been trained to ferment dulcitol rapidly showed great permanency; one such culture transplanted twenty-five times in pepton water during a period

of five months and then plated on neutral red dulcitol agar yielded only fermenting colonies. Twenty colonies from a MacConkey plate of pure typhoid were inoculated into dulcitol broth; the time required for acidity to appear varied from eleven to thirty-two days. Slow fermenters of dulcitol died in dulcitol broth in two months while quick fermenters remained alive longer. He found one strain which did not ferment arabinose, but which after three months' subculturings became a quick fermenter. Three strains that did not ferment glycerol became after eight months' subcultivation quick fermenters, but never in less time than three to four days. In plating out on glycerol media he found a mixture of quick and slow fermenters.

Reiner Müller (1908, 1911) did not observe acid production in rhamnose by any of his typhoid cultures and noticed that the colonies on the rhamnose plates remained small and delicate but that 5 per cent rhamnose produced no more inhibition than 0.5 per cent. He showed, further, that other bacteria of the typhoid-colon group are not inhibited by rhamnose. He observed no production of acidity by typhoid bacilli on litmus agar containing arabinose, dulcitol or raffinose, but he observed acidity on rhamnose Endo agar.

Bull and Pritchett (1916) found an atypical typhoid strain showing irregularity in fermentation, glucose, levulose, and dextrin being all positive and the other sugars negative; indol being positive as in the case of *Bact. coli*, but the organism agglutinated in 1:20,000 dilution.

Krumwiede, Kohn and Valentine (1918) inoculated thirty-seven strains of *Bact. typhosum* into xylose broth and found that twenty-nine produced acid in twenty-four hours while eight of the strains required from five to thirteen days for this result.

Winslow, Kligler, and Rothberg (1919) recently reported the results of similar investigations of various bacteria. They describe the typical typhoid bacillus as a Gram-negative, non-spore-forming rod, actively motile. It forms translucent irregular colonies on gelatin media and faint, nearly colorless growths on potato. It produces strong and prompt acid but no gas in media containing the hexoses, maltose, mannitol, sorbitol, xylose and dextrin; it does not attack arabinose, rhamnose, or lactose; produces a slight initial reddening of litmus milk, which after two weeks reverts to a neutral or slightly alkaline reaction. It

fails to form indol or liquefy gelatin, will not grow in asparagin-mannitol medium, does not reduce neutral red and causes browning of lead acetate media. It has low tolerance for acid, but rather high tolerance for brilliant green dyes and alkali. It has characteristic serum agglutination reactions and is found in human stools and urines as an actual or potential cause of typhoid fever.

OUR OWN WORK ON VARIATION IN THE UTILIZATION OF CARBOHYDRATES

Some of this work has already been reported in the *Journal of Infectious Diseases*, 1920, 26, 52-76. The following table represents a condensed summary of previous work, together with new experiments performed since then:

Sugar fermentation

Arabinose broth

	<i>Number of strains</i>
First series. Meat infusion broth (litmus indicator)	114
Second series. Nutrose broth (phenol red, china blue, indicators).	117
Third series. The same medium	21
Fourth series. Meat infusion broth (phenol red, china blue, indicators)	24
Results were as follows:	
Positive on the second day	2
Positive on the third day	1
Positive on the fourth day	2
Positive on the sixth day	6
Positive on the seventh day	4
Positive on the ninth day	1
Positive on the tenth day	2
Positive on the eleventh day	1
Positive on the fourteenth day	1
Positive on the twenty-third day	1
Positive on the twenty-fourth day	1
Positive on the twenty-eighth day	1
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Positive (total)	23
Negative on the 30th day	279

The proportion of positives is 8.24 per cent against 91.76 per cent of negatives.

Dulcitol

We could not obtain uniform fermentation results with this sugar, despite many attempts. Some strains gave rise to acid at one time and failed to do so at others. On the other hand certain strains gave rise to alkalinity at the first test and in later tests gave an acid reaction. Usually they produced acid in from one to three weeks.

	<i>Number of strains</i>
First series. Meat infusion broth (litmus)	115
Second series. Meat infusion broth (litmus)	57
Third series. Nutrose broth (phenol red, china blue)	21
Fourth series. Meat infusion broth (phenol red, china blue)	29
Fifth series. Meat infusion broth (phenol red, china blue)	139
The results were as below:	
Positive on the fourth day	1
Positive on the sixth day	26
Positive on the seventh day	15
Positive on the ninth day	31
Positive on the tenth day	2
Positive on the eleventh day	7
Positive on the twelfth day	11
Positive on the thirteenth day	9
Positive on the fourteenth day	11
Positive on the fifteenth day	11
Positive on the sixteenth day	3
Positive on the seventeenth day	3
Positive on the nineteenth day	1
Positive on the twenty-third day	12
Positive on the twenty-sixth day	3
Positive on the thirtieth day	9
Positive on the thirty-first day	1
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Total positive on or before the thirty-first day	186
Negative on thirty-first day	175
The proportion of positives is 51.51 per cent against 48.49 per cent of negatives.	

In all of these tests there was a greater tendency for the indicators (litmus, or china blue-phenol red) to become reduced than was evident in arabinose or xylose broth cultures. For this reason it is possible that the percentage of positives may be too low, as all tubes showing reduction were recorded as negative.

Glycerol

Glycerol broth

We prepared 1 per cent glycerol meat infusion broth, using china blue-phenol red indicator.

	<i>strains</i>
First series	123
Second series	10
Third series	10
Fourth series	10
Total	153

All of the cultures produced acidity.

Recently we studied these phenomena with 257 freshly isolated typhoid cultures from typhoid carriers' stools, using two different percentages of glycerol, namely 2 per cent and 6 per cent. We never found any alkali production in the early stage of incubation, but observed acid production between the third and eighth day; later some of the cultures showed reduction of color.

Inosite

Inosite is not a true sugar, but its character is similar to that of sugar and therefore it deserves discussion here.

Inosite broth

One hundred and forty-two strains were inoculated in 1 per cent inosite meat infusion broth, using china blue, phenol red indicator. No fermentation or gas production resulted after thirty days' incubation. Nine subcultures were transferred from inosite broth to inosite broth with the same result.

Raffinose

Raffinose broth

We used 1 per cent raffinose meat infusion broth, with china blue-phenol red indicator. In the first and fourth series, typhoid bacilli did not grow. The media showed slight acidity before

inoculation, but not sufficient to account for the inhibition of growth of typhoid bacilli. We do not know what substance caused this inhibitory action.

In the second series 60 strains and in the third series 21 strains were inoculated; all tubes became alkaline on the second day and showed no fermentation after thirty days' incubation. Of course both sets of media showed a neutral reaction before inoculation. Subcultures from a few of these raffinose broth cultures in raffinose broth again failed to produce fermentation.

Rhamnose

Rhamnose broth

One per cent rhamnose meat infusion broth, with china blue and phenol red as indicators was used for this experiment. One set of tubes was inoculated with 144 strains of typhoid, and another with 19 strains. Both produced alkalinity after twenty-four to forty-eight hours' incubation.

Salicin

Salicin broth

One per cent salicin meat infusion broth, containing china blue and phenol red indicator, was used. Two series of experiments, with 144 strains and with 21 strains were carried out, but no fermentation resulted during thirty days' incubation.

Xylose

We used for the most part 1 per cent xylose meat infusion broth, with china blue and phenol red indicator. During the course of this study we carried on many fermentation experiments, in which we found 8 per cent of 126 strains to be xylose non-fermenters, while the remaining strains all fermented xylose in twenty-four hours. Apparently these 10 negative strains, and in addition 12 strains brought from France by Lieutenant Colwell were found to be slow xylose fermenters. On carrying

subcultures of these strains from xylose broth to xylose broth ten strains (not Lieutenant Colwell's strains) became xylose fermenters in twenty-four hours (or somewhat later) after a few transfers.

CROSS FERMENTATION OF BACTERIA IN DIFFERENT SUGARS

It might be supposed that a single enzyme produced by bacteria when cultivated in sugar media, especially those closely related, such as xylose and arabinose, might ferment more than one sugar. To test this supposition, 6 xylose fermenters, 7 arabinose fermenters, 9 dulcitol fermenters (all fermenting in twenty-four hours) and 6 xylose slow fermenters, 6 arabinose slow fermenters and 7 dulcitol fermenters were inoculated into three sets of sugar media. We could not find any evidence of cross fermentation. After thirty-five days' incubation the fermenter of a given sugar still continued to ferment the same sugar, and no other. Therefore, the enzymes produced by bacteria are, as assumed by many workers, specific for each sugar.

DURATION OF THE FERMENTING POWER

Some strains, which had been artificially induced to ferment certain sugars, maintained their fermenting power after three or four months, or even half a year, although few transplantations were made from one fresh medium to another, this being done in some cases to media containing no sugar. Other strains, however, lost their fermenting power quickly, after only one or two transplantations. Dulcitol fermenters especially are apt to be changeable. Our records show two strains which at first fermented dulcitol in twenty-four hours. Later one of them fermented dulcitol only after four days and the other after twenty-one days. Similarly a strain which at first fermented arabinose in twenty-four hours, later required six days for the fermentation of this sugar.

FERMENTATION REACTIONS UNDER ANAEROBIC CONDITIONS

In the preceding experiments our cultures were grown under aerobic conditions. The following experiments were carried out anaerobically:

Two methods were used in these experiments. One was a combination of exhaustion and absorption with pyrogallol as used in this laboratory for routine work; and the other was the one devised by McIntosh and Fildes.

For the latter method we used a glass jar, connected by means of tubing with a tank of compressed hydrogen, which was inverted in a larger jar filled with water to a level of two or three inches above the inverted jar. The latter is held down by weights when its contents are displaced by hydrogen. Another tube fitted with a clamp connects this jar with the jar containing cultures. When everything is prepared in the culture jar a copper gauze package containing platinum asbestos is heated to redness and placed in the culture jar. The jar is sealed and the clamp released gradually thus allowing the hydrogen to enter the culture jar. The platinum asbestos will act as a catalyser to cause combination of oxygen and hydrogen.

When the oxygen in the jar is exhausted no more hydrogen will enter the jar. Then we seal the glass tubing to prevent the entry of air.

On account of its greater convenience we used the latter method more frequently.

Typhoid bacilli did not grow well under anaerobic conditions; consequently all reactions in sugar media were somewhat slower than under aerobic conditions.

A. Sugar broth cultures

We tried six strains in 0.01 per cent glucose, 1 per cent raffinose, 1 per cent arabinose, 1 per cent dulcitol, 1 and 2 per cent rhamnose, 1 per cent xylose and 2 per cent xylose. The results were as follows:

(a) Glucose

Aerobic: On twenty-four hours' incubation media distinctly showed alkalinity.

Anaerobic: Very slightly alkaline, even after ten days' incubation.

(b) *Dulcitol*

Aerobic: On tenth day, two cultures showed good acidity and on twentieth day four of them showed good acidity.

Anaerobic: Did not show acidity on tenth day; on twentieth day three of them showed slight acidity.

(c) *Rhamnose*

Aerobic: On the fourth day showed good alkalinity.

Anaerobic: On fifteenth day began to show alkalinity.

(d) *Xylose*.

True xylose fermenters ferment in twenty-four hours, under both conditions. The results with four slow fermenters follow:

Aerobic: On sixth day one of them and on the eighth day two strains began to ferment.

Anaerobic: Up to the twentieth day no change in reaction appeared, then the same strains that fermented xylose under aerobic conditions produced very slight acidity.

(e) *Arabinose*

Aerobic: On the third day, they showed alkalinity and on the fifth day one strain showed slight acidity.

Anaerobic: Slight alkalinity persisted until the twenty-first day.

(f) *Raffinose*

Aerobic: On second day showed alkalinity.

Anaerobic: On the eighteenth day showed slight alkalinity. In second series, we tested four strains, and in a third series, four strains on xylose broth but they did not show sufficiently definite changes to be described here.

It will be seen from the above that our own work does not in every way agree with the work of others cited at the beginning of this section. As stated above, we believe that the strains of Klotz, McNaught and Wilson cannot be definitely accepted as having been true typhoid bacilli.

In regard to Mandelbaum's *Bacillus metatyphi* we feel that our results seem to indicate that Mandelbaum was dealing with slow glycerol fermenters rather than with known fermenting strains. We should add, however, that we ourselves never encountered any strains which exhibited the characteristics described by Mandelbaum.

As for the strains of Jacobsen none of our cultures corresponded to these.

As to the results of Penfold and Reiner-Müller, our results agree pretty definitely with those reported by these workers.

No strains corresponding to those described by Bull and Pritchett were met with by us and we were able to find no similar ones described in the literature.

We have confirmed the observations of Krumwiede, Kohn and Valentine, and in addition have shown in this work, as well as in a previous publication with Dr. Teague, that rapid xylose fermenters can be produced from slow fermenters with considerable ease.

Although in almost every respect our work corresponds with that of Winslow, Kligler and Rothberg, they do not entirely correspond with the results of these workers in regard to the action of the typhoid bacillus upon arabinose. Subcultures that ferment arabinose rapidly still retain this characteristic after having been kept on plain nutrient agar for one or two months.

The enzyme produced by a typhoid bacillus from one of the sugars, xylose, arabinose, or dulcitol, may be greatly increased without affecting the production of ferments for the other two sugars. In anaerobic cultures of typhoid bacilli the lack of oxygen supply causes a partial inhibition of growth.

DAUGHTER COLONIES²

Reiner Müller (1908, 1911) first showed that *Bact. typhosum* produced daughter colonies on rhamnose agar. He examined a large number of cultures in this regard and found that they all gave rise to daughter colonies and further that *Bact. typhosum* produced daughter colonies in eight days on agar containing as little as 0.025 per cent of rhamnose and in fourteen days on agar containing only 0.01 per cent. He suggests that the development of daughter colonies on rhamnose agar might be utilized in the identification of *Bact. typhosum*; the results

² The term "daughter colony" is used throughout as signifying the type of secondary colonies arising spontaneously within the substance of the parent colony. The formation of these daughter colonies seems to signify that certain individual cells within the colony acquire the property of utilizing the sugar and therefore growing with much greater speed than the remaining bacteria making up the mother colony.

obtained by Penfold, Saisawa, and by us as far as they go, indicate that he was right in concluding that all typhoid cultures exhibit the phenomenon. Muller and Saisawa found that some other bacteria besides *Bact. typhosum* also give rise to daughter colonies on rhamnose agar.

Penfold (1911) found that the twenty strains of *Bact. typhosum* investigated by him all gave daughter colonies on rhamnose neutral red agar and he noticed acid production in none of the daughter colonies. But after a number of subcultures in rhamnose broth, he obtained a strain which fermented rhamnose. Such a rapid fermenter no longer produced daughter colonies on rhamnose agar and even when it was passed through thirteen generations of pepton water and plated on rhamnose agar it still did not give rise to daughter colonies. He found that the Twort lactose fermenting *Bact. typhosum* and a typhoid culture which had been trained to ferment dulcitol rapidly both produced daughter colonies on rhamnose agar. Three strains inoculated on neutral red dulcitol agar yielded daughter colonies as early as the third day and some of the latter were acid by the fifth day. Some plates showed as low as 2 per cent of colonies with daughter colonies, some as high as 50 per cent. Different plates inoculated with the same culture also showed variations within these limits.

Mandelbaum (1912) observed the production of daughter colonies from *B. metatyphi* on glycerol agar; from daughter colonies were obtained organisms which behaved in all respects like *Bact. typhosum*.

Bernhardt and Ornstein (1913) found colonies on dried agar with irregular outlines, and nucleus forms like anthrax colonies besides the normal typhoid colonies. On cultivating in bouillon this organism produced a film on the surface and was only slightly motile. They did not observe any phenomena of hypo- or in-agglutinability. These types we too have seen on dried plates but they are not real daughter colonies.

Gildermeister's (1913) typhoid strains produced daughter colonies on rhamnose agar except in one strain which had been isolated from a stool. The dysentery bacillus, Shiga-Kruse type and Strong type, *Bact. coli*, *Bact. alkaligenes*, paratyphoid bacilli, Gaertner bacilli, and cholera vibrios did not produce daughter colonies, but *Bact. dysenteriae*, Flexner type, and six out of fourteen strains of the "Y" type produced them. Six passages on ascitic agar or transplants over two weeks in rhamnose bouillon caused typhoid bacilli to grow colonies without daughter colonies on rhamnose agar.

Saisawa (1913) observed daughter colonies of *Bact. typhosum* when plated on rhamnose, dulcitol, arabinose (small ones) or erythrite agar after one week. Also he found daughter colonies on plating on rhamnose agar all of twenty-five strains of typhoid, three strains of Shiga type of dysentery, three strains of Flexner type, thirteen strains of Y-type, two strains of Strong type, two out of three strains of Pseudodysenteriae and none of ten strains of Paratyphoid B, five strains of Paratyphoid A, ten strains of Gaertner's bacillus, three strains of mouse-typhoid and six strains of *Bact. coli*. He could not obtain any variation of typhoid bacilli in culture media containing phenol or malachite green or caffein or by heating at 50°C. for one hour.

Daughter colonies have been observed and studied in connection with cultures of *B. anthracis*, *V. cholerae*, *Bact. coli*, *Bact. dysenteriae*, and other organisms, but they were either caused by sugars not considered in this paper or were not due to sugars at all.

OUR OWN WORK WITH DAUGHTER COLONIES

*A. On sugar media without indicator*³

On the second day or a little later, in an isolated thin colony, one or more very small heaped-up yellow or slightly brownish

³ The sugars which we used for our work were analyzed at the Bureau of Standards, Department of Commerce, at Washington, D. C. The results were as follows:

	per cent	
<i>Arabinose</i>	94.1	
Moisture.....	1.1	Audubon Sugar School, Baton Rouge, La., 62 grams arabinose
Insoluble matter.....	1.4	
Undetermined.....	3.4	
Total impurity.....	4.8	
<i>Rhamnose</i>	89.6	
Moisture.....	9.9	Army Medical Museum, Rhamnose, AMS
Undetermined.....	0.5	
<i>Xylose</i>	100.6	
Moisture.....	0.2	257378, xylose, Difco Standard, Digestive Ferment Co., Detroit, Mich., U. S. A.
<i>Raffinose</i>	82.5	
Moisture.....	15.1	Raffinose—no label to show where from
Total impurity.....	2.4	
<i>Dulcitol</i>		
Polarization.....	0	Dulcitol 10 grams Special Chemicals Co., Not inc., Highland Park, Ill.
Polarization in presence of borax.....	0	
Melting point.....	188	

yellow granules appear on the surface. Their borders can be distinctly seen by means of a hand lens or a low power microscope. Day by day they increase in thickness, in size, and in number, as the mother colonies enlarge. Later a confluent growth of the daughter colonies may entirely overgrow the mother colonies.

B. On sugar plates containing indicators

On plates which contain decolorized china blue the daughter colonies appear, blue in color and inside the mother colony. After a few days they increase in size, color, and number. The blue color doubtless is due to the production of acid by daughter colonies. Ten days or two weeks later, owing to a reduction of the dye, some of the fully grown colonies may have a brownish yellow color. The number of large blue daughter colonies that develop on the plate varies greatly according to the strains of typhoid bacilli employed, there being in some cases only 1 or 2, and in other cases 50 or 100 colonies.

Agglutination tests made with cultures of daughter colonies also showed no differences from those done with the original strain. When we used methylene blue eosin xylose plates (Holt-Harris and Teague, 1916) the mother colonies showed a pinkish color, but the daughter colonies appeared as white dots by transmitted light, some of which soon became black. These colonies exhibited the same rapidity of growth in the succeeding days that was described in the case of the blue daughter colonies on the china blue plates.

When we fished daughter colonies from the above plates and inoculated into the corresponding sugar broth, the latter showed acid production in twenty-four hours. We plated ten typhoid strains on eleven different 1 per cent sugar plates. On arabinose, dulcitol, raffinose, xylose and rhamnose plates from twenty to fifty strains were planted. The cultures were observed for about three weeks.

Below are described the variations in growth exhibited on each sugar medium:

1. *Arabinose*. Thirty-three strains plated on this medium. The daughter colonies appear in two to ten days, each colony usually containing many daughter colonies; a few of the daughter colonies frequently developed into large flat, deep blue colonies; all of the strains tested gave rise to daughter colonies.

2. *Dextrin*. No daughter colonies appeared.

3. *Glucose*. Colonies were smaller and denser than on other plates or control plates and no daughter colonies appeared.

4. *Dulcitol*. We plated forty cultures and daughter colonies appeared within two to five days. One or two opaque brownish yellow daughter colonies in a mother colony in succeeding days would grow so rapidly in size that sometimes the mother colony would be entirely covered. Furthermore, there is a tendency toward color reduction as the growth increases.

5. *Galactose*. Colonies were smaller than on control plain plates and no daughter colonies were produced.

6. *Glycerol*. We used 3 per cent glycerol plates for this purpose and obtained colonies which were very thick, opaque and yellowish brown in color. Plates containing China blue produced pale colonies on the first day, which became deep blue later. The color was reduced by the thick growth. No daughter colonies developed.

7. *Inosite*. No daughter colonies.

8. *Lactose*. No daughter colonies were seen.

9. *Mannitol*. No daughter colonies developed.

10. *Maltose*. There was good growth in point of size and thickness, but no daughter colonies were visible. Twenty strains were plated.

11. *Raffinose*. No daughter colonies developed, but ten days later, they showed papilliform colonies which did not increase in size nor in thickness on further incubation.

12. *Rhamnose*. All thirty typhoid strains which we tested on 1/10 per cent rhamnose plates gave rise to daughter colonies as well as on 1 per cent rhamnose plates. On the latter and on 2 per cent and 3 per cent plates, we could see many large opaque brownish yellow colonies scattered here and there with small daughter colonies and 1 per cent rhamnose Endo plates showed the same appearance.

The best method thus far developed for isolating typhoid bacilli from stools consists in plating upon a lactose medium containing brilliant green and an indicator for acid production. It seemed that a further improvement would be introduced by supplying a positive characteristic to the typhoid colonies instead of relying solely on the absence

of acid production. We attempted to accomplish this result by adding rhamnose (0.1 to 0.25 per cent) to brilliant green lactose agar, in the expectation that the daughter colonies within the typhoid colonies would furnish such a positive characteristic; however, we soon persuaded ourselves that this method has no practical value.

13. *Salicin*. No daughter colonies developed.

14. *Sucrose*. No daughter colonies developed.

15. *Xylose*. Fifty strains were planted. All xylose slow fermenters, thirteen in number, gave rise to daughter colonies on plates containing from 2 per cent to 0.04 per cent of xylose. The rapidly fermenting strains did not give rise to daughter colonies.

Sometimes xylose plates which contain 0.25 per cent glucose or which contained brilliant green eosin (Teague and Clurman) were used without interfering with the growth of the daughter colonies.

16. *Control plain plates*.

Some typhoid bacilli produced papillae-like forms on plain plates two or three weeks later when the plates were nearly dried up; such papillae sometimes occur also on plain plates inoculated with paratyphoid bacilli or *Bact. coli*. They never increase in size or in thickness. Therefore, we could not consider them true daughter colonies.

Under anaerobic conditions, we tested four strains, xylose slow fermenters (Rawling's, C-59, 57 and C-188) on 1 per cent xylose china blue plates; only two strains (C-59 and 57) produced daughter colonies on the eighth day; while under aerobic conditions, all four strains produced daughter colonies within three to five days.

RELATIONSHIP BETWEEN RAPID AND SLOW XYLOSE FERMENTERS

Kowalenko (1910, 1911) obtained *Bact. coli* cultures from Neisser, Massini (1907) and Burk, and tried to separate fermenters and non-fermenters from them after plating on Endo plates, and also from cultures which he isolated from a fever patient's stool on the Endo plate. He always obtained red-colored colonies after plating on Endo plates from a red-colored colony on the Endo plate, but he obtained white colonies and red colonies from a white colony on replating just as we observed above. He reached the conclusion after more study that mutation of bacteria could not be effected by influences from without,

by cultivation at various degrees of temperature, by long cultivation, by the addition of chemical substances, or by passing through the animal body. Saisawa (1913) tried in the same way to separate daughter colonies and mother colonies on plating typhoid bacilli but in vain.

It seemed to us that similar studies made with typhoid bacilli in regard to the separation of rapid and slow xylose fermenters from a single original strain might prove of great interest. Accordingly we made subcultures of two xylose slow fermenters two or three times on plain plates, each time fishing a single colony and planting from the single colony in 1 per cent pepton water. Then from the last suspension of a single colony we plated on 1 per cent xylose plates containing china blue or methylene blue-eosin. At the same time, a loopful of suspension was inoculated to 1 per cent xylose broth containing china blue indicator as a control. Repeated subcultures were made by this method in the hope that after some generations we might get a strain of non-xylose fermenters. We did not obtain such results, however. Charts of the subcultures obtained from the two strains follow:

Strain 57

This strain was always plated on 1 per cent xylose containing methylene blue and eosin.

Day on which acidity appeared in xylose broth

Original—fifth day
 I—fourth day
 II—fifth day
 III—third day
 IV—third day

Arabic numerals = interval in days between the appearance of a colony and subculture from it on 1 per cent xylose media containing indicator.

Roman numerals = number of generations.

We obtained only once a few pink colonies from a xylose fermenter. Thereafter we obtained only black colonies from xylose fermenter plates.

Rawling's

This strain was planted on 1 per cent xylose plates containing decolorized china blue.

Production of acidity

Original—seventh day
 I—sixth day
 II—fifth day
 III—ninth day
 IV—seventh day

We could not obtain any nonfermenter from the cultivation on xylose plates.

From quick fermenters which we had isolated slow and rapid types were readily obtained.

II. VARIATIONS IN REACTION TO SERUM

INAGGLUTINABILITY AND AGGLUTINABILITY OF TYPHOID BACILLI

Agglutinability of typhoid bacilli isolated from specimens (blood, feces, urine, or bile) from the patient may vary greatly, depending to some extent on the number of culture generations for which they have been carried on artificial media. This has been reported by many workers (Förster (1897), Johnson and MacTaggart (1897), Müller, Eisenberg (1903), Sawyer (1912) and others). Thus, lack of agglutinability of the isolated bacilli in early culture generations is sometimes misleading in regard to diagnosis.

Schmidt (1903), for instance, erroneously reported typhoid bacilli as paratyphoid, owing to their inagglutinability. This inagglutinable state, acquired by the bacilli in the human body, can be easily produced by artificial means, such as cultivation on antityphoid serum broth. Such observations were first reported by Ransom and Kitashima, and by Müller. The former observed, in 1898, that the cholera spirillum lost its agglutinability when they cultivated it in anticholera serum, and the latter observed the same phenomenon in typhoid bacilli in 1903. The literature upon this subject is extensive and has been compiled in the articles of Eisenberg, Müller, and others.

As early as 1896 Metchnikoff and Bordet showed that cholera spirilla could partially lose their agglutinability under certain circumstances. Bail (1901) made similar observations with the typhoid bacillus, and Kirstein (1904) showed that cultivation at various temperatures could diminish the agglutinability of bacteria. It has been found, indeed, that organisms isolated from different cases of the same disease often varied considerably in their agglutinability in one and the same immune serum. This was noted by Grassberger and Schattenfroh (1900) in their studies upon anthrax. Bordet and Sleswyk (1910) studying the whooping cough bacillus showed that when a horse is immunized with a whooping cough bacillus which has been grown upon blood media, the serum of this animal will powerfully agglutinate this strain, but possesses little or no agglutinating activity against the same strain habituated to growth on plain agar, an observation which they

interpret as meaning that the agar strain has lost its receptors for the absorption of the specific agglutinin and this inability to absorb agglutinin they, indeed, demonstrated by experiment. Park and his collaborators have studied these relationships particularly with the dysentery bacilli, and Park and Williams (1917) make the following statement:

"The maltose fermenting paradysentery bacillus of Flexner was grown on each of eleven consecutive days in fresh bouillon solutions of the serum from a horse immunized through repeated injections of the bacillus. The solutions used were 15, 4 and 1.5 per cent. The serum agglutinated the culture before its treatment in dilutions up to 1 to 800, and was strongly bactericidal in animals. After the eleven transfers the culture grown in the 15 per cent solution ceased to be agglutinated by the serum and ceased to absorb its specific agglutinins. The cultures grown in the 15 and 4 per cent dilutions of serum agglutinated well in dilutions up to 1 to 60 and 1 to 100, and continued to absorb agglutinins. The recovery of the capacity to be agglutinated was very slow, the cultures being transplanted from time to time on nutrient agar; after growth for sixteen weeks, during which it was transplanted forty-three times, it agglutinated in dilutions of 1 to 200. The culture grown in 4 per cent agglutinated in 1 to 500 dilution, and the one in 1.5 per cent in 1 to 800."

And in their new edition (seventh edition, (1920)) they say:

"The agglutinogenic power, or power to stimulate the production of other antibodies, is not lowered when bacteria become less agglutinable."

The presence of a capsule may interfere with or prevent agglutination. The capsule, developing best in body-fluid or tissues is probably a protective substance. Porges (1905a,b) has outlined a method for the removal of the capsule as a preliminary to agglutination.

Eisenberg (1913) studying a typhoid strain carried along in blood bouillon for a considerable period, found similar development of inagglutinability. And Schmidt (1903) has cited a case of a typhoid bacillus isolated from human disease in which inagglutinability led to prolonged error of diagnosis. Bail working with typhoid bacilli cultivated from the peritoneal exudate of infected guinea pigs showed that under such conditions the organism loses a considerable degree of its agglutinability and attributes this to the development of a capsule-like substance which insulates the bacteria against the antibodies.

A definite loss of agglutinability under similar circumstances was noted by Zinsser and Dwyer (1918) in connection with experiments

upon proteotoxin, but without their finding anything in the nature of a capsule which could explain the phenomenon. Ransom and Kitashima, Müller (1911), Hamburger, Walker (1904) and several other workers have also produced inagglutinability by cultivating on sera containing agglutinin. Porges and Prantschoff (1906) used this method, obtaining irregular results, and attribute this to individual variations in the strains used.

Moon (1911) produced two substrains of a single typhoid bacillus culture by the Barber method, one of which agglutinated with anti-typhoid serum and the other did not. A few generations later both bacilli showed equal agglutinability.

In Zinsser's book (1918) *Infection and Resistance*, he states: "This lessened susceptibility to antibodies is noticeable not only in strains cultivated from the body in disease, but can be produced artificially by cultivating the bacteria on inactivated, homologous immune serum. Such strains may not only increase in virulence, but lose in both agglutinability and susceptibility to bactericidal effects."

Sacquepee (1901) obtained similar variations by keeping the organisms in collodion sacs in the peritoneal cavity. Sawyer (1912) isolated a strain from a typhoid carrier's stool which did not agglutinate with a serum dilution of 1 to 50, but after 11 transplants within two weeks the culture became agglutinable. The same phenomenon has been observed by Scheller (1908).

Recently Gay and Claypole (1913) studying the typhoid bacillus, found that when they produced the carrier state in rabbits the organisms isolated from such rabbits (culturally true typhoid bacilli) failed completely to agglutinate in serum produced with a stock culture and which agglutinated such stock cultures in dilutions as high as 1:20,000. The blood and bile cultures which were inagglutinable by means of the ordinary antiserum, were readily clumped by means of a serum produced by immunizing rabbits with cultures grown on a blood agar medium. If confirmed, these observations, like those of Bordet and Sleswyk, would indicate a complete alteration of antigenic properties by means of cultivation on blood media, and prolonged residence in the animal body; for the serum produced with the cultures upon blood, not only agglutinated these cultures, but also the plain agar growths, and if the cultures on blood were carried along for some generations on plain agar, they again became agglutinable by the serum produced with the plain agar culture. Gay (1914-15) uses this observation to explain the occasional inagglutinability in ordinary sera, of typhoid bacilli recently

isolated from human cases, and emphasizes the diagnostic difficulties which this may occasion. Bull and Pritchett (1916), however, repeating the work of Gay and Claypole found that 25 generations of cultivation in separate series upon plain agar and upon blood agar did not produce appreciable agglutination differences in a typhoid strain. They carried this out with 57 different strains of typhoid bacilli. Nichols too has contradicted the claim of Gay and Claypole that gall bladder infections could be regularly produced in rabbits by injection of typhoid strains grown on blood agar, an observation which would further strengthen the opinion of a fundamental change in reaction to the animal body and its fluids produced by cultivation upon blood constituents. Our own observations (in which we cultivate the typhoid strains upon normal rabbit's serum) also indicate that such a procedure does not exert any appreciable effect upon their agglutinability. Thus the results of Gay and Claypole concerning the inagglutinability of cultures obtained from infected human beings and rabbits would correspond in principle with the investigations of other workers. But the alterations obtained by them by simple growth upon normal blood agar cannot be accepted as conclusive in the light of contradictory results of Bull and Pritchett and of Nichols, and also in the light of our own failure to obtain appreciable changes in strains carried for many generations on normal rabbit serum broth.

It appears from these researches (and many others which might be cited) that the problem has not yet been solved in all particulars. But the general weight of evidence indicates that cultivation in the presence of specific serum antibodies alters the strains in the direction of lessened agglutinability.

The following experiments upon this phenomenon were carried out by us:

Materials used in agglutination tests

1. Broth. This consisted of 0.1 per cent Liebig's meat extract; 1 per cent pepton (Difco); and 0.5 per cent sodium chloride per liter; its reaction was adjusted to pH 7.0. It was sterilized in the autoclave at 15 pounds pressure for fifteen minutes.

2. Antityphoid serum from rabbits immunized against monovalent strain of typhoid bacilli (Rawlings, C-51, no. 3, Cohen, C-188). Each serum titre was 1:10,000 or 1:20,000 for our standard laboratory strains.

3. Normal rabbit serum. Serum obtained from normal rabbits and inactivated at 56°C. for half an hour. About 1 cc. of each medium was placed in small test tubes used for Wassermann work under strictly aseptic precautions, and kept in an ice-chest.

4. The strains used were plated on plain plates three times, and each time one single colony fished to 0.85 per cent sterile salt solution and from this plated on another new plate.

Technique

Stock cultures or newly isolated cultures as described above were suspended in 1 per cent pepton water, and transferred to serum broth or broth; thence retransferred from serum broth to new serum broth or from plain broth to new plain broth, by using a small platinum loop; then incubated at 37°C. Usually after twenty-four hours' growth the culture was plated on plain agar or implanted on slant, using a platinum needle or a very small platinum loop (in the use of the loop we took great precautions against including any serum broth); after standing overnight in the incubator at 37°C. the growth was evenly emulsified in 0.85 per cent salt solution (the bacterial growth always covered the entire surface of the slant). Special care was taken to have the emulsions of the cultures of as uniform a thickness as possible and for this purpose a tube of typhoid bacterial emulsion was kept for comparison.

Graded dilutions of the serum were made with 0.85 per cent sodium chloride solution and ranged from 1:50 to 1:24,300. Half a cubic centimeter of each dilution was transferred to small agglutination tubes and an equal amount of bacillary emulsion was added to each tube and also to a salt solution control.

The results were recorded after two hours' incubation at 37°C. and again after standing overnight in the ice-chest. The controls never showed agglutination. By this method cultures which had been grown on serum were allowed to develop for one generation on agar without serum before their agglutinability was tested. Controls were treated in the same way.

The serum media were occasionally tested for loss of agglutinating power, and were controlled for contamination by plating on plain plates or Endo plates or by inoculation in sugar media, but results were always negative. Such control cultures were made in sugar media (namely, xylose, arabinose, glucose, maltose, mannitol, lactose, sucrose,

rhamnose, raffinose, dulcitol) in broth, in litmus milk, and in 2 per cent glycerol; or plated on Endo plate; always, as stated above, with negative results.

STRAINS MADE INAGGLUTINABLE BY ARTIFICIAL MEANS

I. Antityphoid rabbit serum (Rawling's strain), with a titre of 1:10,000 for the homologous and for other laboratory strains, was mixed in proportions of one part of the serum to four parts of the broth in small test tubes. In the course of the experiments, this serum being exhausted, another antityphoid serum (made with strain C-51) with a titre of about 1:10,000 was substituted.

The same lot of broth was used in the controls.

The stock strains used were "Rawling's," "C-188," and "Cohen." These were prepared in the manner described above and inoculated into the media.

1. Rawling's strain. Within thirty-eight days this strain was retransplanted twenty-four times from one tube to another, in two parallel series, one upon antityphoid serum broth, the other on plain broth. During the first ten days, it was transplanted every day and later at intervals of several days. Agglutinability was tested eight times during this period with the same serum. Chart 1 shows the results.

As is shown by chart 1, the power of agglutinability in dilution of 1:8000 fell to 1:300 after three days, and twenty-four days later, after fifteen transplants in fresh serum media, the agglutinability was recovered. After that there was no remarkable difference between control and serum cultures, although the culture in serum broth was always somewhat lower in agglutinability than the control. The first readings of the serum broth culture after two hours' incubation always showed much lower agglutinability than the control cultures until the expiration of thirty-five days. The astonishing feature of this experiment was the fact that the serum strain seemed to recover its agglutinability after prolonged cultivation on serum, though at first it had lost it. For this reason, on and after the twenty-third day, we used

the other sera "C-51" and "3," for agglutination tests, but did not observe any striking difference between Rawling's serum and the other two sera in agglutinating Rawling's strain.

2. "C-188" strain was tested in the same way as the Rawling's strain.

Chart I

Rawling strain against Rawling's serum antityphoid

Dilution of serum

20,000
10,000
8,000
6,000
3,000
2,000
1,000
900
800
600
400
300
100
50
0

Day of agglutination test at 3 6 10 24 27 33 38
once
Number of transplants 0 2 5 9 15 16 22 24

Marks

— Control culture plain broth culture
— 24 hours reading } serum media culture
- - - - - 2 hours incubation

In this case, we made agglutination tests eleven times within sixty-three days. Its agglutinability fell from 1:8000 to 1:3000 after one day's cultivation in serum media. Three days later agglutination occurred only in dilution of 1:100. Six days later,

it was below 1:50. On the twenty-seventh day it rose to 1:300 and after the thirty-fifth day to 1:20,000. Thereafter, from the thirty-fifth to the sixty-third day, which was the last day of this experiment, the serum strain and the broth control remained entirely parallel. Other sera, C-51 and 3, were frequently used after the twenty-third day but no differences were seen between Rawling's serum and the other two sera.

Chart II

Cohen strain agglutination against Rawling's antityphoid serum

3. Cohen strain was tested in the same manner. We tested its agglutinability twenty-three times within eighty-five days, within which period we made forty-three transplantations. The results are shown on the next chart.

After seventy-one days this strain recovered its agglutinability gradually and at seventy-seven days it had almost completely recovered this property. But readings two hours after incubation were always slightly lower than the twenty-four hours' readings. On the fourth day very slight agglutination in dilu-

tions of 1:50 and 1:100 occurred, visible only with a lens. After that not the slightest agglutination could be seen in any dilution up to 1:50 until the forty-ninth day. On the forty-ninth day slight agglutination was visible by lens up to 1:300 dilution, and on the sixty-fourth day, we could recognize good agglutination in 1:50 dilution of serum. Then within about two weeks it reached almost the highest dilution of the serum attained by the control culture. Nine tests were made with the Rawlings serum. On and after the twenty-third day, we tested its agglutinability five times with C-51 serum as well as with the Rawling's serum. Readings at the end of two hours showed no agglutination for the first four times but agglutination appeared in 1:100 dilution of the serum after the forty-fifth day. In twenty-four hours' readings no agglutination appeared in 1:50 dilution of serum until the twenty-seventh day while on the thirty-third day, and after, it occurred in a serum dilution of 1:900.

During the same period we tested the strain seven times with antityphoid serum (no. 3). Two hour readings showed that no agglutination appeared in 1:50 dilution of serum until the forty-ninth day. On the sixty-fourth day good agglutination appeared in 1:100 dilution. In twenty-four hours' readings until the thirty-third day no agglutination was seen in 1:50 dilution. From the thirty-seventh to the sixty-fourth day it always showed agglutination up to 1:300 dilution of serum.

Agglutinations of control cultures by each serum ranged from 1:8000 to 1:10,000.

In all experiments done with different sera (Rawling's, C-188, and Cohen) when we changed the serum media there was a slight tendency toward agglutination in the first generation on the new serum. After two or three generations in the new serum media this tendency disappeared.

II. We controlled our experiments with normal rabbit serum broth in place of antityphoid serum broth media. Four parts of broth and one part of normal rabbit serum (inactivated by heating at 56°C. for half an hour) were used for this purpose and a series of agglutination tests carried out with the same strains and in the same manner as before.

Agglutination tests of each culture were carried out five times within forty-three days, using Rawling's serum for the tests. Twenty-eight transplants were made during this period.

III. Since the experiments done so far seemed to show a delicate difference between the reactions of individual strains with different antityphoid sera, we decided to repeat them carrying a single typhoid strain both on an homologous serum and on an antityphoid serum produced with another stock strain. For this purpose we immunized two rabbits, in one case using no. 3 culture and in the other C-51. The former is a rapid xylose fermenter and the latter a slow xylose fermenter. We obtained sera which agglutinated our laboratory strains and their own specific strains in dilution of 1:15,000. Cultivation in normal serum in no case changed the agglutinability of the bacilli, which remained parallel in every way to that of the cultures carried on plain broth. Serum media was again prepared (one part serum to four parts broth) and tubed in small tubes. Experiments as described above were then carried out.

(1) Strain C-51. After two days' cultivation both in its own serum broth and in no. 3 serum broth, this strain slightly lost in agglutinability (1:8000). This continued for twelve days, but on the fifteenth day both series recovered agglutinability equal to that shown by the control cultures.

In no. 3 serum broth its agglutinability was lowered slightly more than by growth in the homologous C-51 serum broth, but there was no very considerable difference between them.

(2) Strain no. 3. This strain was tested in the same way as C-51, by cultivation in its own serum broth and in C-51 serum broth. On the third and seventh days inagglutinability on serum no. 3 was most marked (1:100) both for the culture carried on no. 3 serum and for the one carried on C-51 serum. Both gradually recovered agglutinability and reached normal agglutinability on the thirty-sixth day.

When we used C-51 serum for agglutination tests similar results were obtained. On the third day both series reached the maximum point (1:2000) of inagglutinability. Then they gradually recovered, although in this case recovery to normal agglutinability was delayed for twenty-two days.

This experiment showed that typhoid strains lose agglutinability in the same degree, whether cultivated in a serum broth of their own specific immune serum or on a serum immunized against other strains. No difference was observed between rapid xylose fermenters and slow xylose fermenters.

IV. The above experiments were performed with old stock cultures. We next repeated these tests using freshly isolated strains. Two strains (Owen and Boyle) which were obtained from typhoid carriers' stools were used after three generations on artificial media.

The media consisted of four parts of broth and one part of Rawling's or C-51 serum, the agglutinating titre being 1:15,000.

The Owen strain was cultivated in Rawling's serum and C-51 serum broth and transplanted into fresh media six times in seven days. It reached its maximum inagglutinability (1:900) in seven days with the same serum in which it was grown. When tested with the other serum its loss of agglutinability was not so great. A few days later both cultures in Rawling's serum broth and in C-51 serum broth recovered their agglutinability.

Observations were made with the Boyle strain in the same manner as with the Owen strain. In this case the maximum point of inagglutinability in Rawling's serum broth and in C-51 serum broth was reached on the fourth day.

On carrying out agglutination tests with Rawling's serum one strain (Rawling's serum culture) continued its inagglutinability (1:300) at least ten days, while the other culture in C-51 serum broth regained its agglutinability on the seventh day. The other tests with C-51 serum ran almost parallel. The maximum point of inagglutinability (1:900) was reached on the fourth day and agglutinability was recovered on the seventh day.

In the foregoing experiments observations were made concerning the character of the growth of typhoid bacilli in serum broth *in vitro*. During the first few days the bacteria grew like a mass of cotton at the bottom of the tubes; later when the bacteria had partly recovered agglutinability some organisms grew on the surface of the media, forming a film, as well as at the bottom. Up to this point the mass of bacteria is not easily broken up by shaking, but after recovery of agglutinability it becomes very

easy to break up the spongelike growth. A few days later the broth becomes turbid, at first, with a few clumps of agglutinated bacteria, and later only a uniform turbidity is present; in the last stage, at least one month later, the cultures grow slightly more turbid in the tubes, and about two months later, the general turbidity of the cultures is only slightly less marked than that of control cultures in broth.

The experiments just described were carried out in concentrated antityphoid serum media. It seemed important to observe what influence would be exerted on typhoid bacilli by cultivation in broth which contained a very low percentage of antityphoid serum. This might perhaps tell us what influence would be exerted upon the bacilli in the early stages of typhoid fever.

We prepared a number of broth tubes containing Rawling's serum in a proportion of 1:10,000. The titre of this serum against Rawling's culture was 1:10,000. We used two strains for these observations, one the Rawling's strain, the other a culture designated as Sanguist, which had been freshly isolated from a patient's blood.

We carried out agglutination tests eight times within thirty-three days during which we made twenty-two transplants. Rawling's strain showed practically no change, a result distinctly in contrast with its loss of agglutinability when grown in concentrated serum broth. The Sanguist strain diminished in agglutinability after three days although the difference between the serum culture and the control broth culture was not great. After thirty-two transplants thirty-three days later, it regained agglutinability completely. In short, in this experiment a small amount of antityphoid serum in media did not produce any marked change in agglutinability of typhoid bacilli.

When the serum contained blood cells the typhoid bacilli acted haemolytically and reduced the medium from a reddish color to yellow in twenty-four hours.

Several varieties of sugar media and litmus milk were inoculated from the cultures described above and no differences appeared between control cultures and serum broth cultures within three weeks.

AGGLUTININ ABSORPTION TESTS OF XYLOSE QUICK FERMENTER
AND XYLOSE SLOW FERMENTER

The next experiments were undertaken to determine whether xylose quick fermenters and xylose slow fermenters differed in regard to serum reactions.

We immunized rabbits against the xylose fermenting strains "Cohen" and "no. 3," and against the xylose slow fermenters, C-51, Rawling, and C-188. The serum titre of each strain was between 1:10,000 and 1:15,000 for each strain. Three agar slants of xylose quick fermenter twenty-four hours old were prepared and a suspension in 5 cc. of 0.85 per cent salt solution made. To each suspension was added 5 cc. of each corresponding serum in dilution of 1:50. The tubes were then incubated for three hours at 37°C. After that, we centrifuged them for half an hour at high speed, and pipetted off the supernatant fluid. This was then made up with 0.85 per cent salt solution into a series of dilutions ranging from 1:100 to 1:16,200. To 0.5 cc. of each dilution was added 0.5 cc. of the suspension of a xylose quick fermenter, and similarly to another series of dilution, 0.5 cc. of a suspension of a xylose slow fermenter was added. The two series were incubated for two hours at 37°C., and allowed to stand overnight in the ice-chest. The results were read before placing in the ice-chest and again the next morning. As controls, agglutination tests were carried out using the original strains and fresh unabsorbed serums.

After absorption of both sera by slow xylose fermenters we found that practically all agglutinin had been removed. The only exception to this was a slight persistence of agglutinin in the tube containing the 1:200 dilution. Vice versa we absorbed the serum produced with xylose quick fermenters by means of a xylose slow fermenting organism and then looked for traces of agglutinin by adding suspensions of both xylose rapid fermenting and xylose slow fermenting strains. The results were the same as those obtained above.

Control tests gave negative results.

Xylose slow fermenters and xylose quick fermenters are therefore not serologically different.

It is worth noting in this connection that Lieut.-Col. H. J. Nichols tells us that he has found no difference between rapid and slow xylose fermenters in regard to their virulence for rabbits.

Having determined the peculiar conditions under which typhoid bacilli become inagglutinable and subsequently regain their agglutinability, without removal from the specific immune serum, it seemed important to determine whether this was due to inability to absorb the agglutinins or possibly whether it had some relationship to a changed reaction to electrolytes in solution. Also it seemed important to determine whether or not something analogous to an insulating capsule as described by Bail, Kuhnemann and others was responsible for the phenomenon.

Accordingly we first proceeded to carry out agglutinin absorption tests as follows:

a. Agglutinin absorption test

This test was carried out several times using the Cohen strain culture in serum broth for sixty-six days with 49 transplants in serum broth (see chart 2). As controls Cohen and C-188 strains were cultures in plain broth. The serum used was usually Rawling's, sometimes others. The technique employed was that described in the section on agglutinin absorption tests of xylose quick fermenter and xylose slow fermenter. The results follow:

TYPHOID STRAINS BEFORE TREATMENT BY THE SERUM	DILUTION OF RAWLING'S ANTITYPHOID SERUM								SALINE CON- TROL
	100	200	400	800	1,600	4,800	14,150	43,200	
Cohen serum broth culture	—	—	—	—	—	—	—	—	—
Cohen broth cul- ture (control) . . .	+++	+++	+++	+++	+++	+++	++	—	—
C-188 broth culture (control)	+++	++	++	+++	+++	+++	+	—	—

+++ = complete agglutination.

++ = good agglutination.

+ = good agglutination but still cloudy.

— = negative. No agglutination.

Results are recorded after two hours incubation at 37°C. and standing in an ice chest over night. Hereafter we shall use the following abbreviations:

Cohen S = Cohen strain cultivated in serum broth

Cohen B = Cohen strain cultivated in broth (control)

C-188 B = C-188 strain cultivated in broth (control).

Tests done in Rawlings serum after absorption of this serum in dilution of 1:100 at 37°C. for three hours with the "serum" and the "broth" strains respectively:

(a)

BACTERIAL SUSPENSION	DILUTION OF RAWLING'S SERUM PREVIOUSLY TREATED BY COHEN S							SALINE CONTROL
	200	400	800	1,600	3,200	9,600	28,800	
Cohen S	—	—	—	—	—	—	—	—
Cohen B	+++	+++	+++	+++	+++	++	—	—
C-188 B	+++	+++	+++	+++	+++	+	—	—

(b)

BACTERIAL SUSPENSION	DILUTION OF RAWLING'S SERUM PREVIOUSLY TREATED BY COHEN B							SALINE CONTROL
	200	400	800	1,600	3,200	9,600	28,800	
Cohen S	—	—	—	—	—	—	—	—
Cohen B	+	—	—	—	—	—	—	—
C-188B	+	—	—	—	—	—	—	—

Tests with C-188 serum

Before absorption

BACTERIAL SUSPENSION	DILUTION OF C-188 ANTITYPHOID SERUM								SALINE CONTROL
	100	200	400	800	1,600	4,800	14,400	43,300	
Cohen S	—	—	—	—	—	—	—	—	—
Cohen B	+++	+++	+++	+++	+++	+++	+++	+	—
C-188 B	+++	+++	+++	+++	+++	+++	++	—	—

After absorption of C-188 serum in dilution of 1:100 at 37°C. for three hours.

(a)

BACTERIAL SUSPENSION	DILUTION OF C-188 SERUM PREVIOUSLY ABSORBED WITH COHEN S							SALINE CON- TROL
	200	400	800	1,600	3,200	9,600	28,800	
Cohen S	—	—	—	—	—	—	—	—
Cohen B	+++	+++	+++	+++	+++	+++	+	—
C-188 B	+++	+++	+++	+++	+++	++	—	—

(b)

BACTERIAL SUSPENSION	DILUTION OF C-188 SERUM PREVIOUSLY ABSORBED WITH COHEN B							SALINE CON- TROL
	200	400	800	1,600	3,200	9,600	28,800	
Cohen S	—	—	—	—	—	—	—	—
Cohen B	+	—	—	—	—	—	—	—
C-188 B	+	—	—	—	—	—	—	—

*Tests with "Cohen" serum**Before absorption of serum*

BACTERIAL SUSPENSION	DILUTION OF "COHEN" ANTITYPHOID SERUM								SALINE CON- TROL
	200	400	800	1,600	3,200	6,400	12,800	23,600	
Cohen S	—	—	—	—	—	—	—	—	—
Cohen B	+++	+++	+++	+++	+++	+++	+++	+	—
C-188 B	+++	+++	+++	+++	+++	+++	++	+	—

After absorption of "Cohen" serum, diluted to 1:200 at 37°C. for three hours

(a)

BACTERIAL SUSPENSION	DILUTION OF "COHEN" SERUM WITH COHEN S							SALINE CON- TROL
	400	800	1,600	3,200	6,400	12,800	25,600	
Cohen S	—	—	—	—	—	—	—	—
Cohen B	+++	+++	+++	+++	+++	—	—	—
C-188 B	+++	+++	+++	+++	++	+	—	—

(b)

BACTERIAL SUSPENSION	DILUTION OF "COHEN" SERUM WITH COHEN B							SALINE CON- TROL
	400	800	1,600	3,200	6,400	12,800	25,600	
Cohen S	—	—	—	—	—	—	—	—
Cohen B	—	—	—	—	—	—	—	—
C-188 B	+	—	—	—	—	—	—	—

* Slight.

These experiments showed that Cohen S cultures did not absorb the agglutinin of any of the three antityphoid sera.

The work of Bordet has demonstrated the essential importance of electrolytes in agglutination, and the studies of Neisser and Friedemann, Friedberger and others have shown that whereas small traces of salts will flocculate bacteria that have absorbed agglutinin, it requires very large amounts of the same electrolytes to flake out normal (insensitized) organisms. Since the researches of Neisser and Friedemann especially have shown that to a certain extent there is a relationship between the degree of sensitization and the amounts of salt necessary to bring about agglutination, we considered it of interest to determine by comparative tests the differences in sodium chloride concentration necessary to flake out, on the one hand, the Cohen serum strain, and on the other, the Cohen broth strain after both had been treated with homologous serum. This would show whether or not any trace of agglutinin had been absorbed by the Cohen S strain in the process.

Typhoid bacterial suspensions in Rawling's serum were centrifuged for thirty minutes and all the supernatant fluid decanted off for another agglutination test; the residue in both centrifuge tubes, one of them containing Cohen S and the other Cohen B strains was poured into a few centimeters of 0.85 per cent salt solution and without centrifuging but after slight shaking, all the fluid was decanted from the tubes, leaving a mass of bacteria. This was worked into an emulsion by means of a glass rod, and distilled water added drop by drop. The graded dilutions of the 20 per cent sodium chloride were prepared, to range from 20 per cent to 0.025 per cent. A half cubic centimeter of each dilution was transferred to small tubes and a half cubic centimeter of the above bacillary emulsion in distilled water was added to each tube and also to a distilled water control. The results were as follows:

The percentage shown below is the actual percentage after mixing salt solution and watery bacterial suspension:

	10	9	8	7	6	5	4	3	2	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.05	0.025	NO SALT
Cohen S emulsion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cohen B emulsion	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

This experiment shows that the Cohen S strain absorbed either no agglutinin at all or so little that it could not be demonstrated by strong solutions of sodium chloride.

We have now shown that the typhoid bacillus, which loses agglutinability after prolonged cultivation on immune serum, becomes inagglutinable because of its failure to absorb agglutinin. It was next desirable to determine, if possible, whether this inability to absorb antibody was due to an ectoplasmic insulation substance identical with or analogous to a capsule, since capsule formation in other bacteria has been shown to protect against serum effects. In spite of repeated attempts we never succeeded in demonstrating a capsule in our serum strains by staining methods. However, Bail and others have suggested that such capsular materials might be present in bacteria without showing demonstrable morphological change, except perhaps in the form of increased size of the bacterial cell as a whole. For this reason it seemed advisable to investigate this question by the method of Porges, who rendered the heavily capsulated and inagglutinable *Friendländer* bacilli agglutinable by dissolving off the capsule with weak acid and moderate heat.

If it be true that the inagglutinability of Cohen S is due to the formation of a capsule or something similar, then hydrolysis of protein of that capsule by Porges' (1905a, 1905b) method should restore the agglutinability. With this idea in mind, we tried the next experiment, using Rawling's serum, and carrying out the method of Porges exactly as described by him. The following tubes were prepared:

- I. 3 cc. of each bacterial suspension plus 2 cc. of salt solution.
- II. 3 cc. of each bacterial suspension plus 1 cc. of N/4 HCl plus 1 cc. of salt solution.

III. 3 cc. of each bacterial suspension plus 1 cc. of N/4 HCl plus 1 cc. of N/4 NaOH.

To 0.5 cc. of each of these mixtures graded serum dilutions from 1:100 to 1:25,800 were added; also to a salt solution control.

Results were as follows:

SOLUTION	BACTERIAL SUSPENSION	DILUTION OF RAWLING'S SERUM									SALT CONTROL
		100	200	400	800	1,600	3,200	6,400	12,800	25,600	
I	Cohen B	+++	+++	+++	+++	+++	+++	+++	+	-	-
	Cohen S	-	-	-	-	-	-	-	+S	-	-
II	Cohen B	-	-	-	-	-	-	-	-	-	-
	Cohen S	-	-	-	-	-	-	-	-	-	-
III	Cohen B	+S	+S	-	-	-	-	-	-	-	-
	Cohen S	-	-	-	+S	+	+	++	++	+++	+++

+S = slight agglutination.

In another experiment suspension I, II (without the addition of 1 cc. of salt solution) and III (without the addition of 1 cc. of N/4 NaOH solution) were made up. They were heated at 80°C. in a water bath for fifteen minutes, and then cooled in cold water. To no. II, 1 cc. of salt solution and to no. III, 1 cc. of N/4 NaOH solution were added respectively. After being shaken well, 0.5 cc. of each suspension was added to each of the serum dilutions described above.

The results were as follows:

SOLUTION	BACTERIAL SUSPENSION	DILUTION OF RAWLING'S SERUM									CONTROL
		100	200	400	800	1,600	3,200	6,400	12,800	25,600	
I	Cohen B	+	+	+	+	+	+S	-	-	-	-
	Cohen S	-	-	-	-	-	-	-	-	-	-
II	Cohen B	-	-	-	-	-	-	-	-	-	-
	Cohen S	-	-	-	-	-	-	-	-	-	-
III	Cohen B	+	-	-	-	-	-	-	-	-	-
	Cohen S	-	-	-	+S	+	++	++	+++	+++	+++

From these experiments it becomes apparent that the inagglutinability of our serum strains is not analogous to the similar inagglutinability of the Friedländer bacilli used by Porges in his experiments. At any rate our experiments do not permit us to conclude that the acquired inagglutinability of our strains is due to a capsule or to any analogous substance.

Acid agglutination

In 1911 Michaelis (1911) investigated the flocculation of bacteria by acids. His experiments were based upon the fact that serum globulins, casein, the so-called nucleoproteins and other forms of protein could be shown to precipitate at very definite optimum H-ion concentrations. Since Kraus (1897) and Neisser and Friedemann (1904) had claimed that the agglutination of bacteria depended upon the precipitation of a protein constituent of their cell bodies, Michaelis believed that for each species of bacteria an optimum H-ion concentration could be found which would agglutinate them. This he indeed demonstrated, and claimed that the typhoid bacillus especially could be shown to agglutinate at a definite H-ion concentration sufficiently characteristic to aid in the diagnosis of this organism. A number of investigators have confirmed this. For this reason we carried out the Michaelis technique with our broth and serum strains, to determine whether the H-ion optimum for agglutination had been altered by growth in serum.

The solutions were made as follows:

NUMBER	NORMAL SODIUM HYDRATE	NORMAL ACETIC ACID	STERILE WATER
	cc.	cc.	cc.
I	0.5	0.75	8.75
II	0.5	1.0	8.5
III	0.5	1.5	8.0
IV	0.5	2.5	7.0
V	0.5	4.5	5.0
VI	0.5	8.5	1.0
VII			10.0
VII	10 cc. of 0.85 per cent salt solution		

Both Cohen S and Cohen B cultures were suspended in sterile distilled water. Then 0.5 cc. of each of the above solutions and 0.5 cc. of bacterial suspension were put in small test tubes and the results recorded after two hours incubation at 37°C.

	I	II	III	IV	V	VI	VII	VIII
Cohen S.....	—	—	—	—	—	—	—	—
Cohen B.....	—	+S	++	++	+	+S	—	—
C-188 B.....	—	—	+	++	++	+S	—	—
Michaelis results of typhoid bacilli.....	—	+	++	++	+	—	—	—
Bact. coli by Michaelis results.....	—	—	—	—	—	—	—	—

This experiment shows that cultivation in serum has rendered the Cohen strain inagglutinable in an H-ion concentration which agglutinates the normal strain. In this test Cohen S resembles *Bact. coli*, but other tests (sugar fermentation) showed it to be an absolutely typical typhoid bacillus.

Similar tests were made with an inorganic acid, as follows:

HCl agglutination

Suspensions of both strains (Cohen S and Cohen B) were made in sterile water. From normal hydrochloric acid solutions in the following table were prepared by adding sterile water, 0.5 cc. of bacterial suspension, and 0.5 cc. of each acid solution were mixed in small test tubes and incubated at 37° for two hours, then stood in the ice chest overnight. The results were then noted.

The table below shows the final dilution of hydrochloric acid in the combination of acid and bacterial suspension:

	PROPORTION OF ACID											WATER CON- TROL
	10	25	50	100	200	400	800	1,600	3,200	6,400	12,800	
Cohen S.....	—	—	+	++	+++	++++	++++	++++	+	—	—	—
Cohen B.....	—	—	—	—	—	+	++	+	—	—	—	—

Here Cohen S is very agglutinable in the presence of hydrochloric acid, although it is not so in the presence of acetic acid, as we have seen in Michaelis' test. Michaelis himself used only acetic acid.

SUMMARY AND CONCLUSIONS

In the study described above we have endeavored to investigate the variations which may occur in the characteristics of typical typhoid bacilli in regard to their abilities to utilize carbohydrates, and their behavior to serum antibodies under various conditions of cultivation. Underlying our plan of experimentation was the purpose of determining whether variations noted by others and described in many published accounts could properly be regarded as mutations in the botanical sense, and, secondarily, to contribute to the comprehension of the nature and the permanence of variations from type so often noticed in freshly isolated cultures, incidentally thereby shedding some light upon the practically difficult problems so often encountered by the bacteriologist in the identification of strains isolated from the human and animal body.

In order to obtain a true picture of the fermentative characteristics of a given species of bacterium it is clear from the foregoing study that the inoculation of a rather small number of strains into fluid media containing the various sugars and the recording of acid and gas production after a few days' incubation is entirely inadequate. A large number of strains must be investigated and the period of observation extended to thirty days, if necessary. Furthermore, it seems advisable to regard "fermentation" not as the production of gas and acid or even as the production of acid from the sugar by the bacterium, but simply as the utilization of the sugar as a food by the bacterium. Fermentation in this sense is demonstrated quite conclusively on agar plates if characteristic daughter colonies appear on the agar containing the sugar and no daughter colonies appear on control plates of the same agar without the sugar; precisely those cultures that yield unsatisfactory results in broth containing the sugar are apt to give rise to a well marked development of

daughter colonies. The writer believes that this method deserves a much more extended use in bacteriology than it has hitherto received. It seems likely that one could employ it to determine whether other substances besides sugars, for example, certain amino-acids, are utilized by the bacterium in question as a food, and in this way obtain a more accurate knowledge of the metabolism of the organism and possibly important diagnostic distinctions also.

By investigating a large number of strains of *Bact. typhosum*, using broth fluid and solid media and extending the period of observation over several weeks time it was observed that the behavior of many strains toward certain sugars varied widely from that of other strains. The slow fermenters of xylose resemble the mutations of the higher plants first described by de Vries, more closely than some of the other variants considered in this paper because they retain their characteristics quite constantly (for several years at least), so long as they are not grown in media containing xylose; all such strains investigated by us, with one exception, could be trained by long continued cultivation in xylose-media to produce acid in xylose broth in twenty-four hours like the typical *Bact. typhosum*. Furthermore, all of these slow fermenters, including the one that never produced acid in xylose-broth for us, showed daughter colonies on xylose agar; hence we were aware of the fact that these strains could utilize xylose as a food-stuff long before this had become evident from the observation of the xylose-broth tubes.

These observations would seem to indicate that even in the slowest xylose fermenters the xylose utilizing power is potentially retained as a latent characteristic. This would prevent our correctly applying to such cultures the term "mutation" in the sense of de Vries. Working with bacteria we are enabled to observe in a short time a sequence of generations far beyond anything that can be observed with higher plants and our work suggests, though of course in an entirely inconclusive way, that at least some of the "mutations" described by botanists may represent, in fact, a suppression of characteristics which remain latent and might easily become apparent again could a sufficient

number of generations be subjected to an environment in which this characteristic could again become useful.

The inoculation of solid media proved that all of our strains of *Bact. typhosum* utilize both arabinose and dulcitol for their nutrition. In fluid medium nearly half of the strains produced acid in dulcitol in from five to twenty days while only a small percentage showed acid production from arabinose. In striking contrast to the behavior of the typhoid strains in xylose broth, the strains that produced acid in arabinose or dulcitol in one test often failed to produce acid when the test was repeated under similar conditions.

All of our typhoid strains gave rise to daughter colonies on rhamnose agar, but none were observed to produce acid in rhamnose broth.

Our cultures showed no daughter colonies on raffinose agar and did not produce acid in raffinose broth. It is thus apparent that instead of stating that a given species of bacterium ferments such and such sugars, it should be said; that the organism in question can utilize such and such sugars as a food; that the organism produces acid in twenty-four hours in certain of these sugars; that a majority of the strains produce acid after several days in such and such sugars; etc., etc. Having obtained this information concerning certain related species of bacteria it should usually be easy to select those sugars which would yield the most reliable information for the differentiation of the species. Thus, Krumwiede (1918), and quite recently Jordan also, have recommended acid production in rhamnose broth as the most fundamental method of differentiating the paratyphoids from *Bact. typhosum*. We agree that this is a good practical medium for the differentiation of these species but we should bear in mind that *Bact. typhosum*, although it does not show acid production in rhamnose broth, is nevertheless able to utilize this sugar as a food.

Considerable discussion has arisen concerning the question whether certain of the variations from the normal type mentioned in this paper represent true mutations in the sense of de Vries. The writer believes that this term, which defined changes of a

definite character occurring in higher plants should not be introduced into bacteriology; for the bacteriologist, who studies his species not only from the morphological point of view, but also with regard to biochemical and immunological reactions, and who observes not a few generations only, but hundreds and thousands of generations, would almost surely have to modify the conception of the term in such a manner as to cause confusion to the botanist. It, therefore, seems advisable to leave the term mutation to the botanists and, for the present at least, to speak of atypical varieties of bacteria or simply of variants.

Typhoid bacilli grown upon normal serum do not become inagglutinable. Cultivated continuously upon specific immune serum they at first become inagglutinable, but if such cultivation is persisted in for two weeks longer, eventually these strains again become agglutinable. In some cases this return to normal agglutinability does not occur until the seventy-second day. This, however, is exceptional. It is important to notice that, whereas in other experiments the normal characteristics had developed, in this case a return to normal reaction with serum was brought about in spite of a continuous subjection to the unusual environment.

Inagglutinability of the typhoid bacillus is accompanied by inability to absorb agglutinin. There is no difference whatever in these relations between the xylose slow and xylose rapid fermenters. Our experiments do not indicate that capsular material is concerned in the inagglutinability. Treatment by the Porges method does not render the inagglutinable strains agglutinable.

Acid agglutination experiments by the method of Michaelis showed that the inagglutinable serum strain reacted negatively in the increasing concentrations of acetic acid, being in this way similar to colon bacilli, whereas the same strain cultivated in broth reacted with the acid typically as did Michaelis typhoid cultures. In other words, the inagglutinable strains showed a changed reaction in regard to hydrogen ion concentration of the environment.

Finally, we believe that since all the alterations brought about by artificial environment in the typhoid bacillus were rapidly lost when the organisms were returned to the environments prevailing under the usual cultural conditions and in the case of the inagglutinable strains, even in the course of persistent abnormal environment, the changes observed by others as well as by us should properly be regarded as variants and cannot be spoken of with accuracy as mutations in the sense of de Vries.

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