ON A METHOD OF ISOLATING AND IDENTIFYING
BACILLUS TYPHOSUS, BASED ON A STUDY OF
BACILLUS TYPHOSUS AND MEMBERS OF THE
COLON GROUP IN SEMI-SOLID CULTURE MEDIA.

BY PHILIP HANSON HISS, JR., M. D., ASSISTANT IN BACTERIOLOGY, COL-
LEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY;
ASSISTANT BACTERIOLOGIST TO THE NEW YORK
CITY HEALTH DEPARTMENT.

*(From the Bacterial Laboratory of the College of Physicians and Surgeons, and the
Research Bacteriological Laboratory of the Health Department
of New York City.)*

GENERAL CONSIDERATIONS.

The experience of all workers in bacteriology includes instances of the occurrence of unusual variations in the colony and growth characters presented by the same organism when transplanted from one medium to another of apparently the same composition and developed under the same conditions of temperature and moisture. When one considers the crude methods often employed to determine the reaction of a medium, and the changes so readily produced in media, especially those containing gelatin, by the exposure to heat in their preparation, these variations are not remarkable, and probably indicate no especial tendency on the part of the organism to vary, but only its natural appearance under the conditions present. Bearing in mind the value placed upon growth appearances, especially upon the form and size of colonies, in the differentiation of species, one is impressed, in reviewing the literature of bacteriology, with the laxity and paucity in descriptions of the mode of preparation and of the exact composition and reaction of the media in which these appearances occur. The differences in the media used by different observers may account to some extent for the discrepancy in accounts of the same organism and may also be responsible for its description as a new variety or species.

The observations of Baginsky, Rosenthal and Klie are of interest in this connection, especially as they call attention to the variation of colony forms in media of reduced consistence. These experimenters fail to record in detail the reaction of the media used.

Baginsky * in 1889, while examining stools from cases of cholera infantum, noted peculiar colonies showing ramifying outgrowth and forms not usual to *Bacillus coli communis*. From these appearances he thought that he had to deal with an undescribed organism, but careful study upon new plates showed it to be identical with *Bacillus neapolitanus*.

Werner Rosenthal† met with peculiar colonies which he found to be those of *Bacillus coli communis*. He experimented afterwards with the variation in the colony forms of *Bacillus coli communis* and *Bacillus typhosus* in media containing 2.5 per cent and 3.3 per cent of gelatin. He notes that his observations and those of Baginsky were made during the hot months.

Klie‡ continued the experiments of Rosenthal and came to the conclusion that the temperature is of as much if not more importance than the percentage of gelatin, for it has three effects: increase of the rate of multiplication of the bacteria, increase of their motility, and its effect upon the consistence of the medium.

Kruse§ considers that the finer structure of colonies does not show great constancy, and that growth characters depend especially upon the composition of the medium, its consistence, degree of alkalinity, and upon the growth conditions, especially temperature, and he also notes that through a reduction in the consistence of the gelatin, such as is caused by prolonged boiling, colony forms are influenced and "proteus-like" appearances may occur.

From such facts as these it seems fair to conclude that the form of colonies and the distribution of an organism on and in the various culture media are to a great extent purely mechanical problems, that they are the result of the resistance offered by the density of the medium, the motility or non-motility of the organism, and its rate and mode of increase under the given nutrient and temperature conditions.

The influence of the products of metabolism, and of the changes produced in the various constituents of the medium by the organism during growth, must not be lost sight of in this connection, for the presence of these products is often unfavorable for the further development of the organism, and this may show itself more quickly in a solid medium in which these products cannot diffuse rapidly, and become locally more concentrated, than in a fluid or semi-solid medium.

Too much stress therefore cannot be laid upon the necessity of a de-

* Baginsky, *Berlin. klin. Wochenschrift*, 1889, 996.

† Werner Rosenthal, *Deutsch. Arch. f. klin. Med.*, lv (1895), 513.

‡ Klie, Inaug. Diss., Erlangen, 1896, and *Centralbl. f. Baktr.*, xx (1896), 49.

§ Kruse, in Flüggé's *Die Mikroorganismen*, 3te Aufl., p. 480, Leipzig, 1896.

tailed description of the constituents of the medium, the mode of preparation, the reaction and the temperature conditions under which the appearances noted occur, as a variation in any or all of these factors may cause differences in the appearance of the growth or the colony forms.

Having been thoroughly impressed with the importance of the above considerations, and believing that a profitable and scientific field of research lay in the comparative study of bacteria, especially motile forms, in media of carefully varied consistence and composition, obtained by varying the proportion of the constituents of our ordinary nutrient media, *i. e.* the gelatin, agar, peptone, glucose, alkali, acid, etc., the writer some fifteen months ago undertook experiments the results of which led him to the researches described in the present paper.

These researches have been limited to the study of *Bacillus typhosus* and members of the "colon group," and have been carried on in an attempt to establish a broad and practical differentiation between these two forms of organisms, based on their growth peculiarities in semi-solid media, especially in media becoming semi-solid between 30° and 40° C.

The term "colon group" is used broadly and includes members of both the colon and aerogenes groups, described by Kruse in the third edition of Flüggé's "Mikroorganismen." According to Kruse, the most fundamental difference between these groups is the motility of the colon group and the absence of motility in the aerogenes group. Since some of the aerogenes group at times display motility, even this difference between these groups is not constant.

During these experiments it was found that cultures of *Bacillus coli communis* obtained from the different laboratories and other sources were widely different in their behavior in semi-solid media. It has therefore been necessary to divide them roughly into two groups, dependent to a great degree upon their difference in motility.

The absolute identification of the different members of the "colon group" is not a matter of paramount importance to us, since the question under consideration is not so much to differentiate one organism, for instance the true *Bacillus coli communis*, from *Bacillus*

typhosus, as to obtain a differentiation broad enough to include all members of the group which may be found associated with *Bacillus typhosus* in material subjected to investigation.

The cultures of *Bacillus typhosus* and of *Bacillus coli communis* and of members of the "colon group" at my command were from many different sources, some having been cultivated for years on artificial media, others being tested soon after isolation. Thirteen different typhoid cultures have been used uniformly throughout the experiments. Among the typhoid cultures is a culture from Prof. R. Pfeiffer of Berlin, which has been regularly used at the laboratories of the New York Department of Health in the serum diagnosis of typhoid fever, also a culture from Dr. Wyatt Johnston of Montreal used in the same work. Several of the cultures are from the Pathological Laboratory of the Johns Hopkins University and one recently from Kral's laboratory in Prag. Of the other cultures experimented with, one was obtained by spleen puncture, and several were isolated from the organs of patients dead of typhoid fever.

DIFFERENTIATION OF *BACILLUS TYPHOSUS* FROM THE BACILLI OF THE COLON GROUP BY TUBE CULTURE.

NON-MOTILE OR LESS ACTIVELY MOTILE DIVISION OF THE COLON GROUP.

The first cultures of the colon group under observation belonged distinctly to the less actively motile division, and their behavior and appearances in the various media experimented with will be described in detail and compared with the characters presented by the typhoid cultures under similar conditions, and then the active type will be considered.

The preliminary experiments were made with tubes of 10 per cent nutrient gelatin inoculated with *B. typhosus* and *B. coli communis* and placed in the incubator at 37° C. The tubes containing the typhoid growth, examined after 24 hours, showed a uniform clouding of the medium, while the medium containing *B. coli* remained clear, with floculi scattered throughout and a slight deposit. As it seemed probable that these differences were due to the difference in motility of these organisms, a less fluid medium was tried to determine whether changes in consistence would increase these differences in appearance.

For this purpose an agar-gelatin mixture of 0.5 per cent of agar and 8 per cent of gelatin was adopted as the stiffening basis. The first medium of this kind tried contained 0.5 per cent meat extract, 0.5 per cent NaCl, 2 per cent peptone and 1 per cent glucose, and showed a reaction 1.5 per cent acid to phenolphthalein. The inoculated tubes were allowed to remain in the incubator at 37° C. for 24 hours. The cultures of *B. typhosus* showed a uniform clouding of the medium extending completely across the tube and downwards with even base, to a varying extent depending upon the time and depth of puncture, but generally becoming completely clouded within 24 hours. The growth along the lines of puncture was hardly more dense than throughout the rest of the medium. The colon cultures, on the other hand, developed a growth only along the line of inoculation, at times with some outspreading of isolated colonies. Later, in some parts of the medium, a local clouding often developed. Gas was formed, and in the wake of the ascending bubbles streaks and patches of growth occurred, giving in 24 to 48 hours a medium generally clear, with a varying number of these patches scattered throughout it.

The next experiments were made to determine the effect of different quantities of acid and alkali on the growth and action of the organisms, and also with the hope that the local clouding, which displayed itself in some of the colon cultures, would be suppressed by a certain percentage of one of these ingredients.

Fourteen media were made, containing 0.5 per cent meat extract, 0.5 per cent NaCl, 1 per cent peptone and 1 per cent glucose, and varying in reaction from neutral (phenolphthalein) to a reaction indicating 2.5 per cent of normal NaOH solution; and from neutral to 4 per cent of normal HCl solution, by 0.5 per cent increments. (See Table I, p. 683.)

Cultures of *B. typhosus* and of *B. coli communis* in these media showed that the maximum growth for both organisms occurred in media of the same reaction, *i. e.* containing between 1.5 per cent to 2 per cent of normal NaOH solution. In the case of the typhoid bacillus the media giving the optimum growth often showed a complete and uniform clouding within 18 hours, and always within 24 hours. The colon bacillus gave an abundant growth within 24 hours, and a marked outspreading of isolated colonies extending from the line of inoculation. Gas was always produced, and in the track of the bubbles streaks and masses of growth appeared.

As the percentage of alkali decreased or increased from the most favorable reaction, so the growth and rapidity of spreading decreased in the typhoid and the colon series. When the reaction became 1 per cent

acid, only a faint clouding occurred in the upper part of the typhoid tubes, and the growth along the puncture in the colon tubes became very meagre indeed. In both series growth did not take place in the media containing the higher percentages of acid, being generally inhibited in the media containing about 3.5 per cent normal HCl solution.

These media were also inoculated on the surface by means of a loop from broth cultures, and as such surface inoculations gave the most information in regard to the maximum growth and rapidity of spreading, especially with the typhoid cultures, tables recording observations of such development are given (Tables I and II, pp. 683, 684).

It was found by these experiments that any of the media which were uniformly clouded by the typhoid growth served very satisfactorily to differentiate *B. coli* from the typhoid bacillus, yet a tendency was shown by *B. coli*, in parts of the tube, to stimulate the typhoid growth.

Further experiments were, therefore, undertaken to limit if possible this tendency on the part of *B. coli* to cloud the medium, and as all the media heretofore used seemed too complex for definite conclusions in regard to the effect of a given ingredient on the growth of the organisms, the following scheme (Table III, p. 685) was adopted so as to be able to attribute as directly as possible an effect to its proper cause.

This scheme was as follows: The same gelatin agar mixture of 5 gm. of agar and 80 gm. of gelatin to the litre was taken, and to this was added 5 gm. of Liebig's beef extract and 5 gm. of common salt. This was tested, after boiling, for its reaction by means of phenolphthalein, and then brought to the "standard" reaction by the addition of normal NaOH or HCl solution. The reaction adopted as "standard" allows of 1.5 per cent of normal acid, or 15 cc. of normal acid to the litre, over the reaction indicating neutrality to phenolphthalein. This simplified medium brought to the standard reaction was taken as the basis of the series of media.

The series consisted of twenty-nine media. Seventeen of them forming an alkaline and an acid series, graded by differences of 0.5 per cent from standard to 4 per cent of normal NaOH solution, and from standard to 4 per cent of normal HCl solution. Four others of the media were a peptone series, and eight a glucose series (see Table III).

TABLE I.
B. TYPHOSUS, "H." SURFACE-DROP INOCULATION. NOV. 14, 1896. 4.30 P. M.

No.	Water.	Meat extract.	NaCl.	Peptone.	Glucose.	Agar.	Gelatin.	NaOH.	HCl.	18 hours. 37° C.	24 hours. 37° C.	48 hours. 37° C.
1	cc. 1000	gm. 5	gm. 5	gm. 10	gm. 10	gm. 5	gm. 80	cc. 25	cc.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.	
2	"	"	"	"	"	"	"	20		Upper $\frac{5}{8}$ clouded.	Clouded throughout.	
3	"	"	"	"	"	"	"	15		Upper $\frac{4}{5}$ clouded.	Clouded throughout.	
4	"	"	"	"	"	"	"	10		Upper $\frac{3}{4}$ clouded.	Clouded throughout.	
5	"	"	"	"	"	"	"	5		Upper $\frac{1}{2}$ clouded.	Clouded in upper $\frac{1}{3}$.	Clouded throughout.
6	"	"	"	"	"	"	"	neutral.		Upper $\frac{1}{3}$ clouded.	Upper $\frac{1}{2}$ clouded.	Clouded throughout.
7	"	"	"	"	"	"	"		5	Upper $\frac{1}{4}$ clouded.	Upper $\frac{1}{3}$ clouded.	Upper $\frac{5}{8}$ clouded.
8	"	"	"	"	"	"	"		10	Slight clouding in upper portion.	Slight clouding in upper $\frac{1}{4}$.	Upper $\frac{1}{2}$ clouded.
9	"	"	"	"	"	"	"		15	Similar to No. 8, but less.	Same as 18 hrs.	Misty in upper $\frac{1}{3}$.
10	"	"	"	"	"	"	"		20	Similar to No. 9, but less.	Same as 18 hrs.	Slight increase.
11	"	"	"	"	"	"	"		25	Slight growth.	Same as 18 hrs.	Slight increase.
12	"	"	"	"	"	"	"		30	Slight growth.	Same as 18 hrs.	Slight increase.
13	"	"	"	"	"	"	"		35	No growth noticed.	Same.	Slight growth.
14	"	"	"	"	"	"	"		40	No growth noticed.	Same.	No growth.

TABLE II.
B. COLI COMMUNIS, "COLLECTION."—SURFACE-DROP INOCULATION. NOV. 14, 1896. 4 P. M.

Tube No.	Water.	Meat extract.	NaCl.	Peptone.	Glucose.	Agar.	Gelatin.	NaOH.	HCl.	18 hours. 37° C.	24 hours. 37° C.	48 hours. 37° C.
1	cc. 1000	gm. 5	gm. 5	gm. 10	gm. 10	gm. 5	gm. 80	cc. 25	cc.	Some surface growth. No downward extension.		
2	"	"	"	"	"	"	"	20		Surface growth. Slight spreading downward of isolated colonies.		
3	"	"	"	"	"	"	"	15		Surface growth. Some isolated colonies just below surface.		
4	"	"	"	"	"	"	"	10		Same as No. 3. Colonies fewer.		
5	"	"	"	"	"	"	"	5		Same as No. 4. Colonies fewer.		
6	"	"	"	"	"	"	"	neutral.		Surface growth. No colonies.		
7	"	"	"	"	"	"	"	5		Surface growth.		
8	"	"	"	"	"	"	"	10		Surface growth.		
9	"	"	"	"	"	"	"	15		Surface growth.		
10	"	"	"	"	"	"	"	20		Surface growth.		
11	"	"	"	"	"	"	"	25		Surface growth.		
12	"	"	"	"	"	"	"	30		Surface growth confined to original drop.		
13	"	"	"	"	"	"	"	35		Same as No. 12.		
14	"	"	"	"	"	"	"	40		Same as No. 13, but less growth.		
										N. B. Growth less in No. 1 than in No. 2, and decreases from No. 2 as medium becomes more acid.	Practically no change, some slight increase in growth.	Slight increase in growth.

TABLE III.

No.	H ₂ O.	Meat extract.	NaCl.	Agar.	Gelatin.	NaOH.	HCl.	Peptone.	Glucose.
1	1000 cc.	5 gm.	5 gm.	5 gm.	80 gm.	40 cc.			
2	"	"	"	"	"	35 cc.			
3	"	"	"	"	"	30 cc.			
4	"	"	"	"	"	25 cc.			
5	"	"	"	"	"	20 cc.			
6	"	"	"	"	"	15 cc.			
7	"	"	"	"	"	10 cc.			
8	"	"	"	"	"	5 cc.			
9	"	"	"	"	"	standard	=1.5% acid to phenolphthalein.		
10	"	"	"	"	"		5 cc.		
11	"	"	"	"	"		10 cc.		
12	"	"	"	"	"		15 cc.		
13	"	"	"	"	"		20 cc.		
14	"	"	"	"	"		25 cc.		
15	"	"	"	"	"		30 cc.		
16	"	"	"	"	"		35 cc.		
17	"	"	"	"	"		40 cc.		
18	"	"	"	"	"			10 gm.	
19	"	"	"	"	"			20 gm.	
20	"	"	"	"	"			30 gm.	
21	"	"	"	"	"			40 gm.	
22	"	"	"	"	"				5 gm.
23	"	"	"	"	"				10 gm.
24	"	"	"	"	"				15 gm.
25	"	"	"	"	"				20 gm.
26	"	"	"	"	"				25 gm.
27	"	"	"	"	"				30 gm.
28	"	"	"	"	"				35 gm.
29	"	"	"	"	"				40 gm.

No account was taken of any change in reaction caused by the addition of the various percentages of peptone, although the reaction undoubtedly became more acid to phenolphthalein, nor was any account taken of the change of reaction, if any, caused by the glucose, since it was desired to attribute as directly as possible to these ingredients their effects upon the growth and motility, without masking their rôle by the addition of the alkali necessary to bring them back to the standard reaction. By thus reducing the various media to simple terms it has been possible, in a great degree, to attribute directly to the NaOH, HCl, peptone and glucose their effects on the growth, and to select the most favorable elements for the production either of an optimum combination for growth, or a medium serving for purposes of differentiation.

The media were uniformly inoculated from broth cultures, generally 24 hours old; two series, one inoculated by puncture and one by a single drop of the culture on the surface, have always been under observation.

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TABLE IV.

B. TYPHOSUS. "COLLECTION." SURFACE-DROP INOCULATION. NOV. 30, 1896. 4 P. M.
THE NUMBERS CORRESPOND TO THOSE OF TABLE III, GIVING THE COMPOSITION OF
THE MEDIA.

	No.	18 hours. 37° C.	24 hours. 37° C.	48 hours. 37° C.
Alkaline-acid series.	1	Upper $\frac{9}{10}$ clouded.	Clouded throughout.	Same.
	2	Clouded throughout.	Same as 18 hours.	Same.
	3	Clouded throughout.	Same as 18 hours.	Same.
	4	Clouded throughout.	Same as 18 hours.	Same.
	5	Clouded throughout.	Same as 18 hours.	Same.
	6	Clouded throughout.	Same as 18 hours.	Same.
	7	Clouded throughout.	Same as 18 hours.	Same.
	8	Upper $\frac{7}{8}$ clouded.	Clouded throughout.	Same.
	9	Upper $\frac{5}{8}$ clouded.	Clouded throughout.	Same.
	10	Upper $\frac{1}{2}$ clouded.	Upper $\frac{3}{4}$ clouded.	Same.
	11	Upper $\frac{1}{3}$ misty.	Same as 18 hours.	Same.
	12	Slight downward growth.	Same as 18 hours.	Same.
	13	Slight growth.	Same as 18 hours.	Same.
	14	Slight growth.	Same as 18 hours.	Same.
	15	Very scanty growth.	Same as 18 hours.	Same.
	16	No growth.	No growth.	Slight growth.
	17	No growth.	No growth.	No growth.
Peptone series.	18	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	19	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	20	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	21	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
Glucose series.	22	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	23	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	24	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	25	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	26	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	27	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	28	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.
	29	Upper $\frac{1}{2}$ clouded.	Upper $\frac{2}{3}$ clouded.	Clouded throughout.

TABLE V.

B. COLI COMMUNIS. "COLLECTION." SURFACE-DROP INOCULATION. NOV. 30, 1896.
4 P. M. THE NUMBERS CORRESPOND TO THOSE OF TABLE III, GIVING THE COM-
POSITION OF THE MEDIA.

	No.	18 hours. 37° C.	24 hours. 37° C.	48 hours. 37° C.	66 hours. 37° C.
Alkaline-acid series.	1	The NaOH tubes show a slight downward extension of isolated colonies. This increases from tube 1 to tubes 7 and 8 and then steadily decreases as the % of acid becomes larger until tube 17 shows only faintest trace of growth. No clouding occurs.	Increase in growth. No clouding. Same growth proportion between tubes as at 18 hours.	Increase in growth. Otherwise same as 24 hours.	Some clouding in upper $\frac{1}{4}$ of Nos. 1, 2, 4, 6. Increase in downward spreading of colonies in all to No. 11.
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
	11				
	12				
	13				
	14				
	15				
	16				
	17				
Peptone series.	18	Slight surface growth, but no downward extension.	Increase in growth, same as 18 hours.	Some increase in growth.	Slight increase, only slight downward growth.
	19				
	20				
	21				
Glucose series.	22	Slight surface growth. No downward extension. All tubes appear the same.	Same as 18 hours, but increase in growth.	About same as 24 hours.	Same, no downward spreading.
	23				
	24				
	25				
	26				
	27				
	28				
	29				

Alkaline and acid series.—In the case of the typhoid cultures the alkaline media from the one containing 4 per cent of normal NaOH solution to that of standard reaction have generally given uniform clouding within 24 hours when inoculated by a drop on the surface.

The maximum growth and altogether the quickest clouding occurred in media reacting 2 to 3 per cent alkaline over the standard, *i. e.* 0.5 to 1.5 per cent alkaline to phenolphthalein.

In the media to which normal HCl solution was added the growth was scanty in media containing 1 per cent acid, and none was apparent at 3.5 per cent (see Table IV, p. 686).

For the colon cultures the most favorable media were also those of the alkaline series, and in those they showed a downward growth of limited extent and only in mass or by isolated, well-marked colonies, generally giving rise to no clouding until after about 50 or 60 hours. The growth failed in about the same percentage of acid as the typhoid, *i. e.* 3 per cent to 3.5 per cent (Table V, p. 687).

Peptone series.—The peptone media inoculated with the typhoid bacillus became clouded throughout the upper half within 18 hours, and were completely clouded within 42 hours. The growth appeared somewhat denser than in the alkaline media, but the different percentages had no perceptible effect on the amount of growth or the rate of clouding; as is seen by Table IV, all clouded at the same rate.

With the colon cultures (Table V) the growth was less abundant than in the alkaline media, and less spreading of isolated colonies occurred.

The fact that the peptone media of different percentages permitted the same amount of growth to develop in them is worthy of remark, since the reaction as indicated by phenolphthalein becomes acid in direct proportion to the amount of peptone added, and peptone media permitting growth may be much more acid to phenolphthalein than the media of the acid series in which no growth occurs.

Glucose series.—The glucose media were clouded throughout the upper half within 18 hours by the typhoid growth, and completely clouded within 36 to 42 hours. All the media from that containing the smallest amount, 0.5 per cent of glucose, to that containing 4

per cent of glucose, were clouded at the same rate, and the density of growth was about the same. The rate of clouding was slower than in the medium of standard reaction containing no glucose.

Often the growth of the colon bacillus was limited to the original drop, and no growth extended into the medium. No clouding occurred in any of the series. In some cultures of the less active forms of the colon group, especially among freshly isolated cultures, patches and streaks of growth and bubbles of gas were seen throughout the medium after surface drop inoculations; this I believe is due to some actively motile members of the culture wandering out into the medium and there multiplying into isolated colonies and producing gas. This explanation is applicable to the same appearance occurring after puncture inoculation, although here the gas formed by the growth along the puncture passing up in bubbles also permits the formation of isolated patches and streaks in the wake of these bubbles.

It seems probable that the retardation of the clouding in the typhoid cultures and the prevention of clouding and the limitation of growth in the colon cultures in the glucose media are due to the rapid production of acid from the action of the organisms on the sugar.

Puncture inoculations.—In puncture inoculations of both organisms in the same series of media (Table III) practically the same appearances obtained as in the surface drop inoculation series. The typhoid cultures showed the characteristic clouding of the most favorable alkaline media within 24 hours, or even within 18 hours, and the growth along the line of puncture was hardly more dense than that spreading throughout the medium. In the colon series was found the massing of the growth along the line of puncture, and any outspreading was generally by isolated colonies. In the glucose media was seen a more limited growth along the puncture. Gas was produced in the glucose media, and the most characteristic appearance was given by the isolated masses and streaks of growth occurring in the wake of the ascending gas bubbles. No difference in the amount of growth was shown in the various percentages of glucosé; 0.5 per cent seemed to give as much growth and nearly as much gas as the higher percentages.

In the alkaline media, after 50 to 60 hours, a tendency was shown by the colon bacilli to cloud the medium; this was also seen, but to a less extent, in the peptone media, but never did any clouding occur in the glucose media.

One peculiarity of the growth in the peptone media shown by both the typhoid and the colon bacilli, but most markedly by the typhoid, was the early abundant surface growth. This was also at times noticed in the alkaline series, but not to the same extent. The effect of peptone on the growth seems thus to be most marked in the presence of free oxygen.

Reference to the tables, on which are recorded the daily observations, will aid in making clear the above statements (Tables IV and V).

We thus see that in all the media used, with the exception of the acid series, *B. typhosus* gives a characteristic clouding, and always within a short time, while members of the "colon group" show this tendency to cloud only in the alkaline, and very rarely in the peptone media, and these they cloud only exceptionally and then at a late date. This slight tendency of the less motile type of *B. coli communis* to cloud has not been noticed in any of the glucose media. Hence the most favorable medium to be used for differentiation purposes, so far as has yet been ascertained, is one containing no peptone and one or more per cent of glucose and of standard reaction, *i. e.* 1.5 per cent acid to phenolphthalein.

Within 12 to 18 hours the growth in such a medium in tube cultures will present a typical picture; and judging from the behavior of the various cultures subjected to trial, this medium may be depended upon to develop one of the most characteristic points in the differential diagnosis of *Bacillus typhosus* and the less motile varieties of the "colon group."

DIFFERENTIATION OF THE ACTIVELY MOTILE FORMS OF THE COLON GROUP FROM *BACILLUS TYPHOSUS*.

The actively motile forms of the colon group must now be considered. The series of media inoculated with these organisms gave a very different picture from the series inoculated with the less motile

forms. In range and degree of growth in the various media they corresponded closely with the type already described, but in other respects they simulated more the appearance of the typhoid cultures.

In the alkaline series the media were clouded throughout, quite as rapidly as they were clouded by *B. typhosus*, and the cultures could be distinguished from those of the typhoid bacillus only by the greater density of the clouding in the colon cultures. The peptone series resembled the alkaline, but with a more abundant growth on or near the surface.

In the glucose series the growth appearances differed from those in the alkaline and the peptone series. Gas was produced in abundance, and its presence alone served to distinguish the organism producing it from *B. typhosus*. But even after the disappearance of all gas formation, other characteristic differences were present. The media were clouded, with generally a clearer and more fluid stratum at the top, and throughout the media clear spaces occurred, seen best by indirect transmitted light or by holding the tube against the light and slowly passing the finger along the tube between the tube and the light. These clearer spaces were oblong and slit-like and seemed to be caused by the gas bubbles in their passage through the medium. The bubbles might often be seen traveling upward along them in a zig-zag course. Streaks and patches of denser growth occurred also throughout the medium and were often seen along the edges of the clear spaces. The surface of the medium just below the superficial clear stratum had an irregularly fissured appearance which was very characteristic. When such a tube was slanted and the medium allowed to harden at the room temperature, the upper thin slanted portion was often perfectly clear and apparently free from organisms. Sometimes the clear stratum was not well marked.

Experiments have been carried on with the hope, which has so far proved vain, of obtaining a medium which would inhibit or limit the motility of the colon bacilli, and at the same time permit of the characteristic clouding of *B. typhosus*, thereby reducing all the colon growth appearances to one type.

The proportion of the gelatin and agar has been varied in many

ways; for instance, to the litre, combinations of agar 7.5 gm., gelatin 50 gm.; agar 7.5 gm., gelatin 80 gm.; agar 5 gm., gelatin 50 gm.; agar 5 gm., gelatin 100 gm., etc., have been tried, and various sugars and chemicals have been added in different proportions, but these either had no appreciable effect or acted more strongly on *B. typhosus* than on the colon forms.

It thus happens that the medium which was most advantageous for the differentiation of the less motile species also gave the most typical picture for the motile members of the colon group.

It is of interest to note in connection with the behavior of the actively motile bacilli of the colon group in semi-solid media that descendants of the same colony may display differences in motility. This supports the observations made on the inconstancy of the forms among members of the colon and aerogenes groups. In one case a tube of the medium was inoculated from a colony occurring on a gelatin plate made from feces. This medium gave the appearance typical of the actively motile form of *B. coli communis*. Afterwards gelatin plate cultures were made from this growth, and colonies varying from each other in appearance were picked out. Media inoculated from some of these colonies were typical of the active form; those from others of the colonies showed these appearances only in sections of the tubes, and others again gave appearances belonging distinctly to the inactive type.

Behavior of other bacteria in the medium.—No systematic attempt has been made to determine the behavior in the medium of organisms other than *B. typhosus* and the colon bacilli, but sufficient observations have been made to demonstrate that many organisms endowed with motility in no wise simulate the appearances presented by the growth of *B. typhosus*. The absence of peptone and probably the reaction interfere with the ready development of many of these bacteria. Such well known motile organisms as *Proteus vulgaris*, *Bacillus pyocyaneus*, *Spirillum cholerae asiaticae* and the spirillum of Finkler and Prior, give pictures entirely different from that of the typhoid growth. It is worthy of remark that the cultures of the cholera spirillum and the spirillum of Finkler and Prior at my com-

mand are well differentiated from each other by their growth in this medium.

Uses of the medium.—The practical application of the medium to the work of the isolation of *B. typhosus* from suspected material will be considered later.

In making cultures from the organs of cases of suspected typhoid fever, almost positive evidence of the absence of *B. typhosus* is given if the typical clouding does not occur; if it does occur, further tests are of course necessary. If clouding with gas formation takes place, a colon form is probably present, and *B. typhosus* may or may not be present also.

It was thought when rapid clouding of semi-solid media by the typhoid bacillus was first noticed that an easy and rapid means of separation had been discovered, and that by inoculating fairly thick layers of the medium in flat-bottom flasks or in Petri dishes by a single drop, in the centre, pure cultures of *B. typhosus* could probably be obtained from the peripheral zone of the clouded area; but the first trials with artificially infected water and feces demonstrated that other organisms present spread quite as rapidly as the typhoid bacillus, and that this method could not be depended upon. These actively motile bacteria were later found to be the active forms of *B. coli communis*.

DIFFERENTIATION OF THE COLONIES OF *BACILLUS TYPHOSUS* FROM
THE COLONIES OF THE COLON GROUP BY PLATE CULTURE.

In the medium containing 5 gm. of agar, 80 gm. of gelatin, 5 gm. of meat extract, 5 gm. of NaCl, and 10 gm. of glucose to the litre, the colonies of *B. typhosus* were readily distinguished from the colonies of the non-motile forms of the colon group.

The deep typhoid colonies had no definite outline, and decreased gradually in density from the centre to the periphery; the bacilli spreading through the medium from the colony centre, just as they spread and cloud the medium from the drop or puncture inoculation in the tube. Non-spreading forms rarely occurred. Surface colonies were not observed. This was probably due to the ease with

which the bacilli disseminated themselves throughout the medium, and hence did not become heaped into a surface colony as a less motile organism would.

The non-motile colon forms gave rise to large, roughly spherical, deep colonies, with well defined outlines. These colonies were of a light yellow color when seen with the unaided eye; by transmitted light under low power they were generally of a dark greenish brown. Spreading by means of minute isolated bands of bacilli was seen in some instances. Surface colon colonies were fairly numerous. As seen under low power by transmitted light, they were yellowish brown and granular. They were round or irregular, and generally spread out in a fairly thick layer from the dark colony centre.

When the actively motile colon forms came under observation a more difficult problem presented itself. These forms were found not only to form spreading colonies, simulating the typhoid colonies, but often to cloud rapidly the entire plate and to render it impossible to obtain pure cultures from other colonies in the plate. Many expedients were resorted to in order to limit this spreading, and at the same time to allow characteristic colonies of *B. typhosus* to develop. The percentages of agar and gelatin were varied, as was also the percentage of glucose, and in addition to these changes the effect of various chemicals was tried, but with the same result as in the tube experiments; the typhoid organisms were more influenced in their development or limited in their motility by these agents than the colon forms.

Finally, after a long series of experiments, a medium was devised, composed of 10 gm. of agar, 25 gm. of gelatin, 5 gm. NaCl, 10 gm. of glucose and 5 gm. of meat extract (Liebig's) to the litre, and of a reaction indicating 2 per cent of normal acid, phenolphthalein being the indicator used. This medium was found to limit almost entirely the spreading of both the typhoid and the colon bacilli, and at the same time to differentiate the colonies of *B. typhosus* from those of the colon group. As seen under a low power, the deep typhoid colonies are small, generally spherical, with a rough irregular outline, and by transmitted light are of a vitreous greenish or yellow-

ish green color. The most characteristic feature consists of the well defined filamentous outgrowths, ranging from a single thread to a complete fringe around the colony. The young colonies at times are composed solely of threads. The fringing threads generally grow out nearly at right angles to the periphery of the colony, and can be seen by focusing up and down to be directly connected with it. Often there is an angular outgrowth from which the thread arises. The threads may be short, but are often long, and at times even twisted on themselves. A small percentage of the larger deep colonies show no threads. The surface typhoid colonies are small. They usually have a well-defined darker central portion, resembling the deep colony, from which threads may spring, and the part spreading over the surface of the medium is very thin and colorless by transmitted light.

The deep colon colonies are, on the average, much larger than the deep typhoid colonies; they are spherical or of whetstone form, and by transmitted light are darker, more opaque and less refractive than the typhoid colonies. By reflected light, to the unaided eye, they are pale yellow. The surface colon colonies are plentiful, large, round or irregularly spreading, and as seen by transmitted light are brown or brownish yellow. They often show a dark well-defined central spot, and from this spread out in a fairly thick layer over the surface. Their color and size readily differentiate them from the typhoid colonies.

The foregoing descriptions refer to colonies not over 24 hours old and developed at 37° C. Plates should be observed after 16 or 18 hours, for the younger the typhoid colonies the more typical is the thread formation. In old plates the colonies become more dense and approach more closely the colon type, while the older colon colonies may show outgrowths and slight spreading.

If threads occur in the colon colonies, as they in very rare instances do, they are likely to be parallel to the periphery of the colony, and even if at right angles to it, it is generally impossible to determine that they arise directly from the colony. In such a case the large size and the color of the colony, the presence of isolated foci of bacilli,

which are not uncommon around the older colon colonies, and the impossibility of making out the direct joining of the thread and colony, are generally sufficient to prove such a colony not to be typhoid.

In examining plates by the unaided eye the typhoid colonies may often be found without difficulty. They appear as mere greyish points among the much larger and yellowish colon colonies.

The reaction of the medium is most important, and should be as acid as above described. If it is more alkaline the colony appearances of both *B. typhosus* and colon bacilli change distinctly. In a series of experiments with this medium, to which different amounts of acid and alkali had been added, differences were observed in the shape, size and number of the colonies. In the most favorable medium for growth among both the typhoid and colon forms, the surface colonies became very large and spreading, some of the colon cultures giving rise to very large surface colonies, and the typhoid colonies spreading out into thin transparent films of irregular outline. The deep colonies were increased in size and the typhoid colonies lost their thread formation. As the reaction became acid the surface colonies became more regular, rounder and smaller, and the typhoid surface colonies scarcer. The deep typhoid colonies again became typical and showed thread formation. When the medium became very acid the growth was found again less characteristic and the number of colonies distinctly less.

CONSTANCY OF THE DIFFERENTIATION.

In these experiments, extending over many months, there has never occurred among the numerous organisms examined a culture of *B. typhosus* that did not present the typical appearance of the large majority of its colonies, nor has there been observed a culture among the various members of the colon group which gave colonies resembling the typical thread-forming typhoid colony. At times colonies are seen which under a low power resemble the typhoid colonies closely, but higher magnification will generally demonstrate the absence of well-defined threads.

No difficulty has been experienced in selecting the typhoid colonies in plates made from mixtures of the various typhoid and colon cultures under observation. The immediate source of the culture does not seem to affect its colony characters. Colonies of *B. typhosus* from young and old broth cultures, from agar cultures of various ages, and colonies formed by organisms for many days in water, have been found typical, and finally typical colonies develop in plates made from typhoid stools and often in large numbers.

PRACTICAL APPLICATION OF THE COMBINED PLATE AND TUBE METHOD
FOR THE ISOLATION AND IDENTIFICATION OF *BACILLUS TYPHOSUS*.

The method found to be most advantageous in isolating *B. typhosus* is as follows: It consists in making an inoculation in the tube medium (agar 5 gm., gelatin 80 gm.) from the suspected colony. If the growth in this medium is typical of typhoid, then the usual differential tests for the identification of this organism may be tried, but if the appearance is not typical the organism may without hesitation be eliminated as not typhoid. Up to the present time the writer has not met with an organism other than the typhoid bacillus giving the same combined colony and tube characters in these media as those given by *B. typhosus*, so that he has come to consider these characters in combination as almost diagnostic.

The tube medium gives us more information than a simple fermentation tube, for we can not only determine that we have an organism which does or does not produce gas, but we can tell something of the motility; and if the appearance given is typical of typhoid, we know that the organism producing it is either *B. typhosus* or one of the very few organisms which may perhaps give identically the same appearance in this medium. These organisms must be rare, for in all the feces and waters subjected to test none has been found giving an appearance not readily distinguishable from that given by *B. typhosus* at 37° C. It is thus seen that by this process of elimination the number of organisms to be subjected to ultimate tests is reduced to the minimum.

Since it is necessary to allow the plates to remain in the incubator

at 37° C. for only 16 to 18 hours, and since the growth in the tube medium takes only the same length of time to become characteristic, it follows that by this combined use of the plate and tube media we may obtain within 36 hours almost positive evidence that the organisms tested are or are not typhoid bacilli.

Such a plating medium, which permits of the development of characteristic typhoid colonies at incubator temperatures, presents a distinct advantage in water investigations, since high temperatures kill or prevent the development of the large majority of water organisms.

Further, liquefying organisms cannot spoil the plate, and the plates are only in rare instances overgrown by rapidly spreading forms (hay bacillus group?) and then only after many hours.

Although no suspected water has been subjected to test, still it has been found possible to recover the typhoid organisms without difficulty from artificially infected tap-water. In one experiment of this nature three two-litre flasks filled with tap-water were employed. The first was inoculated with two loops of a 24-hour broth culture of *B. typhosus* and six loops of a broth culture of the colon bacillus. This flask was well shaken, and one cc. of the water carried over into the second and from this one cc. into the third flask. 0.25 cc. of the water from each of the three flasks was plated and the plates allowed to remain in the incubator at 37° C. for 20 hours. Inoculations were made in the tube medium from typical colonies. These were found in plates from the first and second flasks only, plates from the third flask giving no typical colonies; but of twelve colonies selected from the plates from the first and second flasks, eleven gave the typical clouding in the tube medium, and only one (which was noted at the time of fishing as probably contaminated) gave clouding with gas formation. The eleven gave all the reactions of the typhoid bacillus.

In the stools examined, many colonies have necessarily been tested, in fact all colonies thought to resemble in any particular those of the typhoid bacillus, either in size, color or by the presence of definitely marked threads (though not otherwise characteristic), yet in no case have the organisms proved to be typhoid which did not present a typical colony, *i. e.* in color, size and thread formation.

That other forms presenting threads may occur in water and feces is to be expected, but, as stated above, planting in the tube medium, so far as the writer's experience goes, has always served to differentiate them from *B. typhosus*.

PREPARATION OF THE MEDIA.

The preparation of the media presents practically no difficulty, and if one uses an autoclave the media may be ready for tubing in an hour and a half. If the agar has to be melted in an ordinary steam sterilizer a longer time is required. The tube medium, consisting of 5 gm. of agar, 80 gm. of gelatin, 5 gm. of beef extract (Liebig's), 5 gm. of sodium chloride and 10 gm. of glucose to the litre, is made by dissolving the agar in 1000 cc. of water to which has been added the beef extract and sodium chloride. When the agar is completely melted the gelatin is added and thoroughly dissolved by a few minutes' boiling. The medium is then titrated to determine its reaction, phenolphthalein being used as the indicator. The requisite amount of normal HCl or NaOH solution is added to bring it to the desired reaction, *i. e.* a reaction indicating 1.5 per cent of normal acid. To clear the medium, add one or two eggs, well beaten in 25 cc. of water, boil for 45 minutes and filter through a thin filter of absorbent cotton. Add the glucose after clearing.

The medium used for plating contains 10 gm. of agar, 25 gm. of gelatin, 5 gm. of beef extract, 5 gm. of sodium chloride, and 10 gm. of glucose. The method of preparation is the same as for the tube medium, care being taken always to add the gelatin after the agar is thoroughly melted, so as not to alter this ingredient by prolonged exposure to high temperature. The glucose is added after clearing. The reaction of this medium is most important, and if, as is usually the case, it is too alkaline, it is rendered of the desired reaction by adding the determined amount of normal solution of HCl. The medium should never contain less than 2 per cent of normal acid.

SUMMARY AND CONCLUSIONS.

Semi-solid culture media, and more especially media rendered semi-solid by temperatures of from 30° to 40° C., seem to have an im-

portant bearing in the differentiation of bacterial species, particularly those presenting various degrees of motility.

In such media not only the effect of differences in consistence on the motility of an organism may be noted, but the effect produced by various chemicals and nutrient ingredients on the growth and motility may be readily observed.

By systematically varying the constituents of such media it has been possible to produce a medium in which the behavior of *Bacillus typhosus* differentiates it from the various members of the colon group; and also to produce a medium in which the colonies of *Bacillus typhosus* assume a form which distinguishes them from the colonies of the colon bacilli in plate cultures.

Bacillus typhosus alone of all the organisms investigated during these experiments has displayed both the power of giving rise to thread-forming colonies in the plating medium and that of the uniform clouding of the tube medium, hence these two characters may prove to be of great value in the identification of this organism.

The practical application of the use of these media has led to the ready detection of *Bacillus typhosus* and its isolation from the stools of patients suffering from typhoid fever.

No suspected water has been subjected to test, but from the investigation of artificially infected tap-water the media here described may be assumed to have an application in the detection of *Bacillus typhosus* in such waters.