A CORRELATION STUDY OF THE COLON-AEROGENES GROUP OF BACTERIA, WITH SPECIAL REFERENCE TO THE ORGANISMS OCCURRING IN THE SOIL

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From the time of the discovery of *Bacterium coli* and *Bacterium aerogenes* by Escherich this group of bacteria has been of profound interest to bacteriologists and sanitarians. As these organisms were first isolated from the human intestine, their presence elsewhere has generally been taken as an index of fecal pollution. They were later found, however, to be normal inhabitants of the alimentary tract of lower animals covering a wide range of species. The problem became still more complicated when it became known that coli-like organisms are widely distributed in nature, particularly in soil and on plants and cereals.

For a time it appeared as if the presence of an organism of the *B. coli* type in water or any other article of human consumption was of no sanitary significance. The subject was so confusing that one German school of sanitarians entirely discarded the colon test. In spite of this situation, the test for *B. coli* has been, and is today, strongly advocated and supported by sanitarians in America, England and France.

Numerous attempts have been made to devise an acceptable system of classification of the colon-aerogenes group, but with very few exceptions these have failed because they did not rest on a sound basis of natural relationships. In the light of recent researches it becomes quite apparent that difficulties in previous classifications were in large measure due to a lack of delicate and exact methods, neglect in the consideration of normal habitat, and faulty interpretation of results. New methods of study have aroused new interest in this group, however, so that within a

brief period of but a few years most important advances in our knowledge have been made. Some of the methods of earlier investigators have given place to newer procedures which rest on broader scientific foundations, as for example the differentiation of types by exact methods of determining the hydrogen ion concentration in media of known composition, and the quantitative relationship of carbon dioxide to the total gas volume. Again, older methods which had been practically discarded have been re-applied and made to serve as very important differentiating tests, as for example the Voges and Proskauer reaction.

In view of the claims held by many investigators that certain types of colon bacilli met with in water have their origin in soil, their occurrence in water being viewed only as the result of soil washings, etc., the question of relationship of types becomes all the more important. Correlation of characters with source, according to such claims, is a problem which requires a most careful and thorough solution, and if true correlation can be established, careful colon bacillus typing will be an essential part of sanitary water analysis.

Both *B. coli* and *B. aerogenes* were long regarded as being of fecal origin. Booker (1891), Hammerl (1897), Hellström (1901), McConkey (1905 and 1909), Clemesha (1912), Rogers and his co-workers (1914–16), Levine (1916), and others showed that the aerogenes type is relatively infrequent in human and animal dejecta, as compared with *B. coli* itself. Winslow and Cohen (1918) estimate the frequency of *B. aerogenes* in animal feces as 0 to 2.6 per cent.

The aerogenes type of colon bacilli appears to be widely distributed in nature, having as a rule a saprophytic existence. Laurent (1899) thought that *B. coli* may lead a parasitic existence on the potato. Klein and Houston a little later (1899–1900) reported the occurrence of both typical and atypical *B. coli* on grains of various kinds. Papasotiriu (1901) obtained results similar to those of Klein and Houston. Prescott (1902 and 1906) showed that coli-like organisms could be found on grains whose contamination with fecal matter seemed very remote. Düggeli

(1904) obtained a large number of similar bacteria from fruits, plants and seeds, and Metcalf (1905) observed the same kind of organisms on flowers, fruits and grains. Bettencourt and Borges (1908) found 12 strains of lactose-fermenting organisms on vegetables and cereals, but only half of these were typical *B. coli.*

McConkey found atypical colon bacilli to constitute 56.2 per cent of the 121 strains isolated from raw water. Houston showed that 13 per cent of his 243 strains from raw water, 5.3 per cent from stored water and 3.2 per cent from stored and filtered water, were atypical *B. coli.* More recently Rogers and his associates (1915–1916) found about 91 per cent of the bacteria on grains, and 33.3 per cent of those in water to belong to the group of non-fecal coli-like bacteria. Their investigations were soon followed by those of Rogers, Clark, etc., Hulton, Greenfield, Levine, Burton and Rettger, Winslow and Kligler, and others.

Houston (1897–1898), studied a large number of soil samples, and came to the important conclusion that true colon bacilli are rarely found in virgin soil, but are present in large numbers in soils that have been grossly polluted with animal matter. Konrich (1910) drew the same conclusions from his examination of 547 samples of soils. Both of these investigators failed, however, to differentiate typical *B. coli* from the non-fecal type (aerogenes-cloacae). Johnson and Levine (1917) found that the aerogenes-cloacae types are the predominant bacteria of this group in the soil. Burton and Rettger (1917) examined 1000 samples of soil, leaves, flowers, etc., and concluded that the cloacae type predominated over its close allies, which is in harmony with the results of Clemesha, who observed that this organism could be isolated readily from soils.

Conflicting reports on the distribution of coli-like bacteria in nature have appeared, however. Clark and Gage (1903) obtained negative tests with grains. Gordon (1904) was unable to isolate lactose fermenters from bran except that of inferior quality. Winslow and Walker (1907) failed to find them in a thorough search in 178 samples of grains and 40 samples of grasses. Neumann (1910) experienced the same difficulty with fresh fruits, but found them on fruits and others foods which had been exposed to human contamination.

The present investigation was planned with the following points in view:

1. To determine the relative frequency of the colon and aerogenes types of bacteria in soils which from all appearances are free from animal pollution.

2. To ascertain whether or not there is a definite correlation between types of bacteria and their origin.

3. To make an extended correlation study of the coli and the aerogenes types of gas-fermenting organisms with reference to some of the most important reactions, particularly the gas ratio, and the methyl red, Voges and Proskauer, and the uric acid tests; and to determine the relative value of these tests in identification work.

4. To determine the relative value of the following media in the present correlation study: (a) The dipotassium phosphate-glucose-peptone (Witte) medium of Clark and Lubs; (b) the same medium with American brands of peptone in place of the Witte; and (c) the synthetic medium of Clark and Lubs.

5. To ascertain whether the coli and aerogenes types of bacteria change their character under prolonged cultivation in a new environment.

METHOD OF COLLECTING SAMPLES

The samples were collected from four main sources in the vicinity of New Haven, Conn., at different seasons and over a period of two years. These sources are East Rock, West Rock, Mt. Carmel and the New Haven Water-shed. In most instances the samples were taken on the summits of the rocks or hills, and in places far remote from human habitations. Chance contamination from birds and wild animals cannot be excluded, however, except perhaps when the samples were taken from underneath precipices and rocks. Thorough sanitary surveys were always made of the surroundings.

Three types of soil were chosen: (1) those from open precipices, (2) from between and under large stones, and (3) from well-aerated open ground. They were selected in such a way as to minimize as far as possible all chances of contamination.

The collecting outfit consisted of a large test tube and a strip of tin one end of which was hammered into a spoon shape. The tin was about an inch longer than the tube, in which it was kept until the time of sampling, both being protected by a cotton plug. This outfit was sterilized in the hot air sterilizer. In taking a sample of soil the dry surface layers were usually removed by a sterile knife and 10 to 15 grams of the soil collected in the tube by means of the tin spatula. The samples were taken to the laboratory immediately.

Four different amounts of each sample were employed in the isolation process, namely 0.01, 0.1, 0.5 and 1.0 gram portions. At first these amounts were weighed out separately in watch glasses, but subsequently they were approximated by comparison with portions of known weight which were used as standards. The different portions of soil were vigorously shaken with definite amounts of water in dilution bottles. The shaking was continued until a uniform emulsion was obtained. After standing long enough for the heavy particles to settle out the supernatant fluid was drawn off and plated on plain agar. The direct plating method for isolation was employed throughout the investigation, in preference to the liquid sugar medium enrichment method. Although the latter method has often been employed, it was feared that its use would disturb the original numerical relationships of the bacterial types.

Litmus lactose agar was at first used in the plating, but was soon found to be very unsatisfactory. Very few red colonies were observed on the plates, and at times not a single red colony was discernable in as many as 20 to 30 plates after forty-eight hours of incubation. This was in harmony with Burton and Rettger's observations on the cloacae-aerogenes type of bacteria, and those of Ayers who showed that alkali-forming bacteria in milk would rarely be noticed on litmus lactose agar.

The plain agar plates were incubated at 30°C. for forty-eight hours. At the end of this period all of the coli-like colonies, noted on examination with the low power objective, were subcultured in lactose fermentation tubes. These tubes were incubated at 30°C. from two to five days, at the end of which time

a sorting out process was conducted. All tubes which failed to show gas production by the end of the fifth day were discarded. The tubes which contained gas were further plated on plain agar, for the purpose of more complete isolation or verification of purity of the cultures. Stock cultures were finally made from well-isolated colonies.

SOURCES OF SAMPLES		NUMBER OF SAMPLES	NUMBER OF SAMPLES YIELDING COLI-LIKE COLONIES	NUMBER OF CULTURES FROM POSITIVE SAMPLES	
				Aerogenes type	Coli type
Soils					
East Rock soil	A	23	7	29	1
	B	25	11	22	1
	C	14	5	20	1
	D	29	13	26	12
West Rock soil	A	23	6	31	0
	B	15	7	13	0
	C	18	11	29	0
	D	8	5	23	5
Water-shed soil	A	19	9	37	0
	B	28	15	40	0
	C	11	4	19	0
Mount Carmel soil	A	12	9	36	0
	B	15	3	37	0
	C	26	6	24	0
	D	6	4	14	0
	E	19	5	27	0
	F	20	7	20	0
Total		317	127	447	20
Feces					
Human (7)		7	7	0	41
Monkeys (2)		2	2	0	30
Horses (4)		4	3	0	31
Cows (3)		3	2	0	23
Sheep (4)		4	4	0	24
Fowls (3)		3	2	0	24
Total		23	20	0	173

For an organism to be admitted into the final collection of strains the following recognized characteristics were required. They must be short rods, staining readily with the ordinary dyes but not by the Gram method. They must fall in the class of non-spore-forming organisms, and possess the ability to attack lactose with the formation of acid and gas.

Altogether 467 strains were obtained from the various soil samples. Of this number 447 were finally identified as belonging to the aerogenes-cloacae subgroup or type, while the remaining 20 were designated as typical *B. coli.*

For comparative study the same types of organisms were sought in human and animal feces, and 173 coli-like organisms were isolated from the feces of 7 men, 2 monkeys, 4 horses, 3 cows, 4 sheep and 3 fowls. No organisms of the cloacae-aerogenes type were found, all of the isolated strains proving to be typical *B. coli.* This should not be regarded as evidence that the aerogenes-cloacae type is not present in the intestines of man and animals. It does indicate, however, that they are of uncommon occurrence there.

The table on page 258 contains a summary of all the samples taken from soil and human and animal feces, showing the number of strains obtained and the types of organisms isolated from each source.

MORPHOLOGY, STAINING PROPERTIES AND MOTILITY

When grown on plain agar the large majority of the strains were alike within narrow limits, and resembled in size and form ordinary *B. coli,* being distinctly rod-shaped, with rounded ends. Some strains were thicker than others, and frequently short forms were observed which were more or less coccus-like. All strains were Gram-negative, although some took the counterstain more deeply than others. All took the ordinary stains readily. Spore formation could at no time be demonstrated.

Motility studies were made on twenty-four hour cultures, grown in Clark and Lubs' medium, and for the greater part of the time by the hanging drop method. The Hesse method of cultivation in semi-solid agar was also employed. While in

many instances motility could be detected without any difficulty, it was at times very difficult to reach a satisfactory conclusion. The following table summarizes the results.

Motility

TYPES OF ORGANISMS	HANGING DROP METHOD		HESSE METHOD		NUMBER EXAMINED
	Motile	Non-motile	Motile	Non-motile	
B. coli	119	54	121	52	173
B. aerogenes	122	325	75	372	447

ACTION ON CASEIN, GELATIN LIQUEFACTION AND INDOL PRODUCTION

The reaction of the different strains in litmus milk was more or less uniform at the end of the observation period, namely seventy-two hours. Some difference was observed, of course, in the rapidity with which visible acid production was brought about; but with few exceptions all of the strains turned the litmus red and coagulated the casein within seventy-two hours. In the exceptional instances application of heat was necessary to bring about the coagulation.

Gelatin liquefaction was determined by the two following methods. In the first, tubes of nutrient gelatin were inoculated (stabbed) with a twenty-four hour agar culture and incubated at 20°C. for six weeks. Observations were made at the end of 3, 7, 14, 21, 39 and 42 days. In the second method, a loopful of a twenty-four-hour broth culture was spread over the surface of standard nutrient gelatin and incubated at 37°C. for thirty days. At the end of five-day intervals the tubes were placed in the refrigerator. Tubes in which no liquefaction was demonstrable were returned to the incubator. The results obtained by both methods are given in the following table.

The superiority of the 37° method is clearly brought out here. According to this, 17 of the aerogenes strains were liquefiers. None of the coli cultures possessed this property.

Gelatin liquefaction

ORGANISMS TESTED	20°C.		37°C.		NUMBER EXAMINED
	+	−	+	−	
B. aerogenes from soil.....	0	447	17	430	447
B. coli from soil.....	0	20	0	20	20
B. coli from feces.....	0	173	Not tested		173

The tests for indol were made by the Salkowski and by the para-dimethyl-amido-benzaldehyde method of Ehrlich (1901). In the former procedure 0.5 cc. of a 10 per cent solution of sulphuric acid and 0.5 cc. of a 0.01 per cent solution of potassium nitrite were added to 5 cc. of a five-day old culture which had been incubated at 30°C. The Ehrlich test was conducted by adding to the same amount of culture material 0.5 cc. of a 2 per cent solution of para-dimethyl-amido-benzaldehyde in 95 per cent alcohol and then concentrated hydrochloric acid drop by drop and in such a way as to keep the two layers of fluid separate. The medium employed in both tests consisted of 10 grams of Witte's peptone, 5 grams of sodium chloride, 0.2 grams of dipotassium phosphate, and 1000 cc. of water. The results of these tests are given in the following table.

Indol production

ORGANISMS	EHRLICH METHOD		SALKOWSKI METHOD		NUMBER OF STRAINS TESTED
	+	−	+	−	
B. aerogenes from soil.....	141	306	80	367	447
B. coli from feces.....	173	0	Not tested		173
B. coli from soil.....	15	5	15	5	20

The marked difference between the results obtained by the two methods with the aerogenes strains from soil is noteworthy, and is due either to the extreme sensitiveness of the Ehrlich reagent, or to the fact that the Salkowski method is in reality not an indicator of indol, but of some other reacting substance, as has been claimed by Kligler (1914) and others. All of the fecal strains of *B. coli* were indol-positive.

CARBOHYDRATE FERMENTATION

A. Acid production

The production of acids and gas by intestinal bacteria was observed by Buchner as early as 1885. He found that the main end products of sugar decomposition by his "Darmbacillus G" were carbon dioxide and fatty acids. Since this time the fermentation reaction in a culture medium has been extensively utilized for the purpose of characterization and classification of bacteria. Its principles have been so firmly established that it is considered one of the very important reactions in the differential studies of organisms of more or less similar as well as widely-divergent types.

At first the acid test was limited to a narrow field, namely, the separation of the colon from the typhoid type of bacteria, as is well illustrated in the early use of the litmus-lactose agar of Wurtz (1892); but at the present time no systematic study of an organism is complete without a resumé of its fermentative properties if it is found to have such. Our chief interest in this paper is, of course, in the colon-aerogenes group of bacteria.

In 1893 Theobald Smith separated the colon bacilli into two groups, according to their action on sucrose. The work of Durham (1900), however, inaugurated the real beginning of the use of the fermentation method in systematic classification. He employed glucose, lactose and sucrose, and characterized *B. communis verus* as glucoso-lactoso-non-sucroso fractor, *B. coli communior* as glucoso-lactoso-sucroso fractor, and *B. lactis aerogenes* as polysaccharid fractor. Ford (1901) chose the same sugars for his classification. He divided the colon group into *B. coli*, *B. lactis aerogenes*, and *B. cloacae*.

McConkey (1905) divided the lactose-fermenting group into the four well-known divisions, based principally upon the action on sucrose and dulcitol, namely: (1) sucrose – dulcitol – (*B. acidi lactici* type), (2) sucrose – dulcitol + (*B. communis* type), (3) sucrose + dulcitol + (*B. communior* type), and sucrose + dulcitol – (*B. aerogenes* type). This classification for a long time served as a frame-work for investigators in this field. Wins-

low and Walker (1907) found that raffinose is generally attacked by sucrose-positive organisms.

Bergey and Deehan (1908) also adopted McConkey's classification, and by adding other cultural tests extended the grouping in a most bewildering manner. They derived 16 groups and 256 varieties. In 1909 McConkey again subdivided his four main groups by introducing more fermentable substances. Over 100 types were separated out in this way.

Jackson's classification (1911) was a modification of McConkey's four primary divisions. He divided these into 16 distinct types by the additional use of raffinose and mannitol. This was formally adopted in 1912 by the Committee on Standard Methods.

Rogers, Clark and Evans (1914) concluded from their study of the colon-aerogenes organisms in milk and milk products that the acid production from a fermentable substance can not be used to advantage. They claimed that the measurement of the gas ratio is far more reliable and constant than the titratable acidity. In his careful study of the acid production of the colon group in many different sugars and under varying experimental conditions Browne (1914) came to the conclusion that the degree of acidity produced by the various members of the colon group is directly proportional to the complexity of the sugar fermented, and that each type within the group has its own limit of acid toleration. One interesting fact brought out is that strains of colon bacilli isolated from feces produced more acid in fermentable sugars than those which were isolated from oysters.

Kligler (1914) studied 80 laboratory strains of *B. coli* and concluded that salicin fermentation offers a better basis of classification than dulcitol. He thus modified McConkey's scheme as follows: Sucrose − salicin + type (*B. communis*), sucrose + salicin − type (*B. communior*), sucrose + salicin, + (*B. aerogenes*) and sucrose − salicin-(*B. acidi-lactici*). Glycerol was further used, to differentiate *B. aerogenes* from *B. cloacae*, the latter being a non-glycerol fermenter.

Rogers, Clark and Evans (1915) again maintained that "the acid from the fermentation of sugar may be masked by a second-

ary alkali production sufficient in some cases entirely to obscure the acid formation." They, furthermore, discredited the salicin test on the ground that too large a percentage of their own cultures (94.6 per cent) fermented this substance.

Levine (1916) maintained that the origin of a strain correlates better with sucrose than with the sucrose-dulcitol fermentation. In his second paper (1916) he concluded that "quantitative acid production in glucose, galactose, maltose, lactose, sucrose, raffinose, salicin, inulin, mannitol, dulcitol and glycerol is not a reliable index for differentiating colon-bacillus-like bacteria." He believes that gas formation is of more value in classification.

Murray (1916) attempted to differentiate human, bovine and equine types of colon bacilli by means of quantitative acid production. He came to the same conclusions as Levine. "In all cases," he says, "the average acid production for each of the 100 strains of each type resembles that of every other, and also resembles the average acid production of all the strains taken together irrespective of origin."

Hulton (1916) studied 45 strains of colon bacilli from various sources and found that a better correlation was obtained between sucrose fermentation and source than between sucrose-dulcitol fermentation and source, confirming Levine's observations.

It appears very clear, from the above cited observations, that acid production, even when determined by the biometric method, does not furnish a sound basis for the classification of the colon-aerogenes group of bacteria. The relationship between the various cultural and physiological characters and the normal habitat of the organisms studied has been in the past lamentably neglected. This important phase of classification has recently been brought to light, however, by Rogers (1914–1916) and his associates. The grouping of the colon bacilli has by them been greatly simplified and placed upon a more natural and logical foundation. In their papers dealing with the bacteria isolated from milk and milk products, feces and grains, they gave us a sound criterion for the separation of the colon group into two distinct types. This separation was accomplished by the determination of the gas ratio in a glucose medium.

The fallacy of the titrimetric method of determining the amount of acid production by bacteria, and the necessity of the application of more accurate and dependable methods for estimating the H ion concentration in culture media and in bacterial cultures have been clearly demonstrated by Clark and by Clark and Lubs (1915–1917). As a result of their important observations, the colorometric method which has been so advantageously applied by others to biochemical problems in animal physiology has quite generally become a routine part of bacteriological procedure.

B. Hydrogen ion concentration and the methyl red test

The successful attempts of recent years to establish more definite relationships of the important members of the colon group with each other and to place their classification on a more scientific basis were stimulated by the works of Harden (1901 and 1905), Harden and Walpole (1906), and Thompson (1911). They found that the end products of glucose fermentation, such as lactic, succinic, acetic and formic acids, and ethyl alcohol, by *B. coli* differ in quantity from those which are formed by the organisms now known as *B. aerogenes* and *B. cloacae*. Michaelis and Marcora (1912) gave further evidence to show that there is a "physiological constant" in *B. coli* cultures when this type of organism is grown in lactose broth. This constant is evidenced by the cessation of its activity at a definite hydrogen ion concentration of 1×10^{-5}.

The Clark and Lubs (1915) phosphate-glucose-peptone medium serves admirably to apply the above principles to practical use. This, as well as the subsequent synthetic phthalate medium of these investigators, furnishes the necessary conditions to permit an indicator of the right choice to register the hydrogen ion concentration within sharply defined limits, so that the distinguishing character of the indicator is not altered or obscured. Their selection of methyl red as the indicator of merit has also done much to facilitate our study of these organisms.

Clark and Lubs found that *B. coli* produced definite changes in these media, with sufficient acid production to give a brilliant red coloration when a few drops of the methyl red indicator are added, whereas a similar test on cultures of the *B. aerogenes* type resulted in a yellow coloration. More than this, they showed that these reactions correlate in a perfect manner with the source of the strains, and with their gas ratio. These observations were soon followed by fruitful researches of other investigators, and were in a large measure corroborated by Levine, Winslow and Kligler, Greenfield, Fulton, Johnson, Burton and Rettger, and Winslow and Cohen.

There are, however points still undetermined in the methyl red test which require further elucidation. Clark and Lubs emphatically state that the use of peptone other than Witte's would lead to an erroneous reaction. With this brand of peptone there are doubtful reactions which have been termed by Clark and Lubs as the "neutral tints." The typing of bacteria by means of the methyl red test depends upon gradations in color varying from a yellow to a bright red color, and covering a range of 4.2 to 6.0 on the pH scale. Determining the hydrogen ion concentration is comparatively easy when pronounced shades of red are obtained on the addition of the indicator; but when the hydrogen ion concentration is so low that only a yellow color ("neutral tints") is produced by the methyl red, it is imperative that some sort of a scale or limit within the "neutral tints" be established, or the types may be incorrectly placed and the correlations disturbed. Levine (1916) in his first work on the methyl red test encountered this difficulty; and Burton and Rettger (1917) found that the variation in the high ratio cultures was such as to lead them to the conclusion that the biometric method was of little value in the classification. Winslow and Cohen (1918) encountered this difficulty in the Witte peptone-phosphate-glucose medium, as well as in the synthetic medium later devised by Clark and Lubs (1916).

EXPERIMENTAL

In the present investigation the methyl red test has been placed on a more strictly quantitative basis, and exact pH values have been given to the various shades of color that were obtained at different times, both in the "neutral tint" range as well as where the characteristic light rose to deep red colors occurred.

In view of the fact that the inconsistent results obtained by some investigators were attributed by some to the indiscriminate use of different brands of peptone, two well-known products were employed, namely Witte's and Difco. The composition of the medium was the same as that first recommended by Clark and Lubs (0.5 per cent each of di-potassium phosphate, peptone and glucose). The new synthetic medium of Clark and Lubs was also used, for comparison.

No definite standards as to the temperature and incubation time for the methyl red test seems thus far to have been agreed upon by different workers. Clark and Lubs chose 30°C. and an incubation period of three to five days, whereas Levine maintained that no difference could be observed whether the temperature was 30 or 37°C. He held that 3 days' incubation is sufficiently long if the temperature is 37°C. More recently, Johnson and Levine (1916) resorted to forty-eight hours' incubation at 37°; Burton and Rettger (1917), five days at 37°; and Winslow and Cohen (1918), four days at 37°C.

Preliminary studies showed that a large number of the soil organisms refused, at least for a while, to grow at 37°C., especially in the synthetic medium, thus apparently confirming the findings of Rogers and his co-workers, and those of Winslow and Cohen. Throughout the present study the incubation temperature was 30°C. Since there was still a question as to the most desirable incubation period to be employed in order to obtain the most uniform and dependable results, both a three and a five day period were given a lengthy trial with all three of the media.

The synthetic medium had a distinct advantage over the others in being practically if not entirely colorless after sterilization, and hence not obscuring the color reactions. However,

after some preliminary attempts to obtain colorless peptone media, a method was followed by which the peptone-phosphate-glucose medium could be prepared and sterilized so that it remained as free from color as the synthetic. Previously sterilized test tubes are filled with the required amount of medium, and the final sterilization carried out by placing the tubes in containers which have impervious bottoms and sides, as for example canning tins or glass beakers, instead of the usual wire baskets, and sterilizing in the autoclave for ten minutes at an extra pressure of 10 pounds. During the entire course of the investigation very few tubes were found not to be sterile. We made it a common practice not to employ the tubed medium until its sterility had been tested by proper incubation for at least twenty-four hours.

The colorimetric standards were prepared according to the descriptions of Clark and Lubs. Three color indicators were employed, namely methyl red, brom thymol blue and brom cresol purple. Brom thymol blue is particularly well adapted for registering the hydrogen ion concentration of aerogenes cultures. The brom cresol purple was selected because it gives more distinguishable shades of color than the methyl red at the less acid end of the methyl red range where the troublesome neutral tints occur. The following technique was adopted for the test.

Five cubic centimeters of the bacterial culture fluid were transferred to test tubes of uniform diameter, and 0.2 cc. of the indicator added which by the preliminary drop method on a watch glass, or better still on a sheet of glazed paper or cardboard, was found to cover the indicated hydrogen ion range. The tubes were sorted out into three groups, with a fair knowledge of the approximate range of hydrogen ion concentration of each group. The pH value of each culture was recorded by comparison in the comparator with the previously prepared color standards.

A total of 3725 individual hydrogen ion concentration determinations was made in the three media with the two types (coli and aerogenes) isolated from soils and feces. The results are shown in the accompanying charts, in which the frequencies of occurrence are plotted as ordinates, and the corresponding pH values as abscissae. The middle line in the charts is an arbitrary

border or methyl red line to separate the coli from the aerogenes types of organisms. Since the colon type is now accepted as methyl red positive, and since the pH value 6.0 is the end point of the methyl red range, this line which starts at pH 6.0 appears to be the natural line of demarcation between these two types, and to be of value in plotting the charts.

Chart I gives the results of the hydrogen ion concentration determinations of the 447 aerogenes strains grown in synthetic and Witte's peptone media for three days at 30°C. Both curves

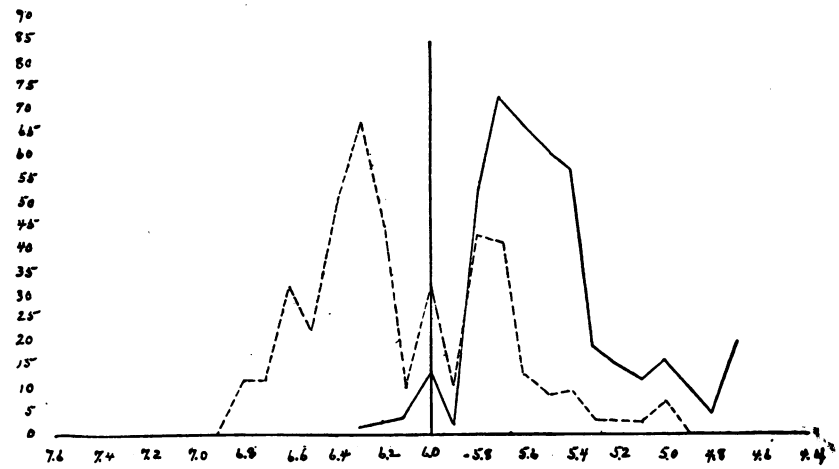

CHART 1. FREQUENCY CURVES FOR B. AEROGENES TYPE (SOIL)

Ordinate represents numbers of strains and abscissa the pH values. Three days' incubation at 30°C. Unbroken line, synthetic medium; broken line, peptone-glucose-phosphate medium (both of Clark and Lubs).

show clearly that a three day period of incubation is not sufficient at this temperature for the methyl red test, especially in the synthetic medium, in which almost all aerogenes cultures become methyl red positive. The peak of the curve for the synthetic medium is located at pH 5.7 and even with the clark medium, a large number of aerogenes cultures enter the methyl red range, although not as many, as in the synthetic medium.

Chart II shows the results of five days' incubation in the three media. There is a striking difference between the results of the

three and five-day periods. The additional forty-eight hours' incubation has returned all of the aerogenes strains to their proper indicator ranges in the synthetic as well as in the Witte peptone medium. The peak of the curve in the synthetic medium is now located at pH 6.5 instead of 5.7, the whole curve covering a pH scale of 6.0 to 6.8. In the Witte peptone medium the pH scale extends from about 6.0 to 7.6, whereas the peak lies at 6.8.

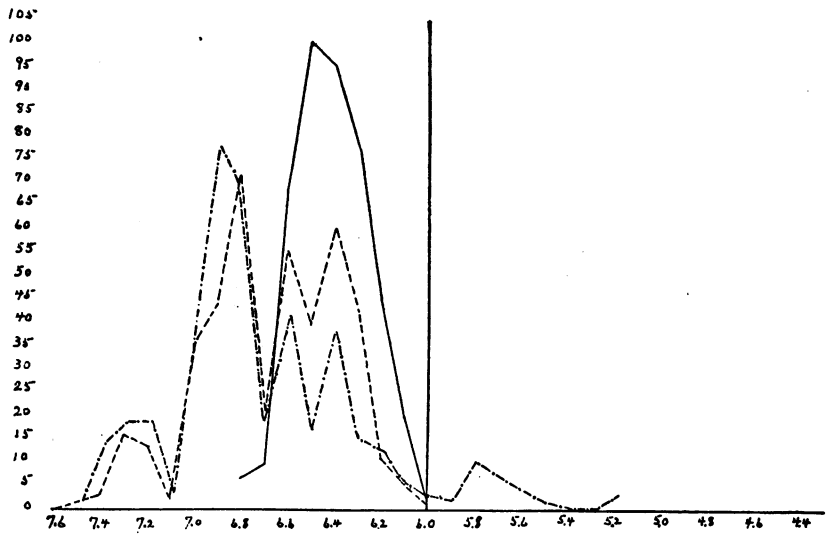

CHART II. AEROGENES TYPE (SOIL)

Five days' incubation at 30°C. Unbroken line, synthetic medium; barred line, Witte peptone-glucose-phosphate medium; barred and dotted line, Difco peptone-glucose-phosphate medium.

When substituting Difco peptone for the Witte in the peptone-phosphate-glucose medium, certain discrepancies occurred, even after prolonged incubation. It is seen that 26 of the aerogenes strains entered the methyl red positive range.

Chart III gives the results of 20 coli cultures isolated from soil, and grown in both the synthetic and the Witte peptone media for three days at 30°C. Chart IV gives the results of the same 20 coli strains in all three of the media after an incubation period of five days.

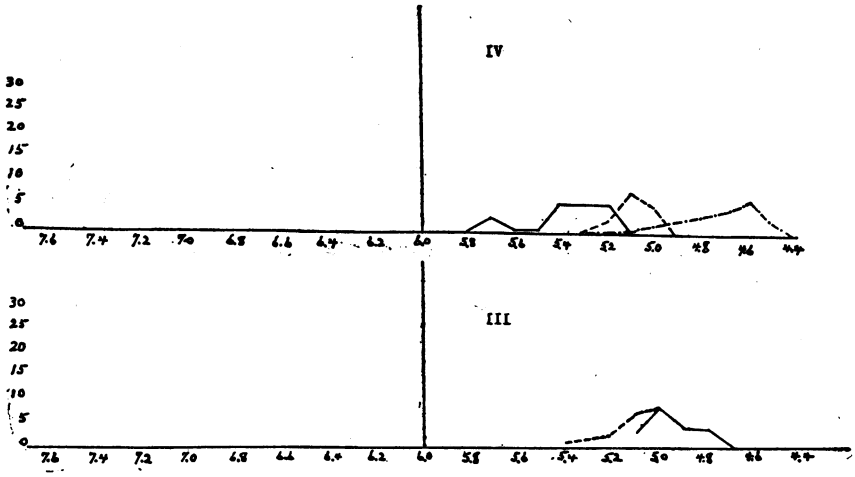

CHART III. (BELOW). B. COLI TYPE (SOIL)

Three days' incubation at 30°C.

CHART IV. (ABOVE). B. COLI TYPE (SOIL)

Five days' incubation at 30°C. Unbroken line, synthetic medium; barred line, Witte peptone-glucose-phosphate medium; barred and dotted line, Difco peptone-glucose-phosphate medium.

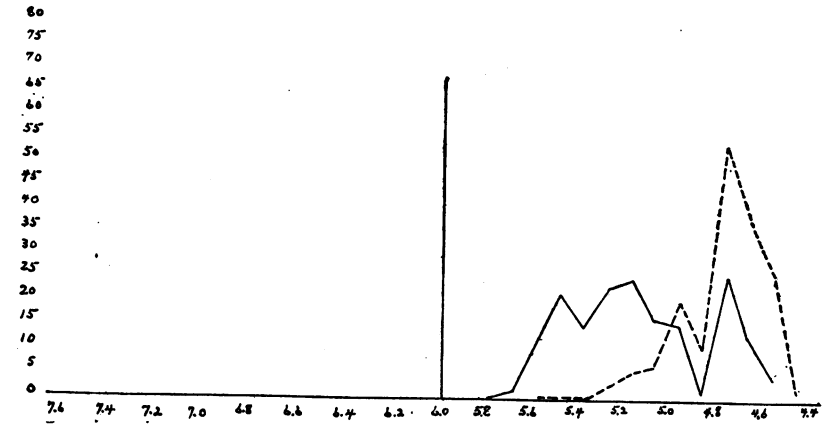

CHART V. B. COLI TYPE (ANIMAL SOURCE)

Five days' incubation at 30°C. Unbroken line, synthetic medium; broken line, Witte peptone-glucose-phosphate medium.

Chart V is plotted with two curves representing 173 coli strains isolated from animal feces. These are the results of the final hydrogen ion concentration determinations in the synthetic and the Witte peptone media after five days at 30°. Again the coli cultures gave uniform results with the methyl red tests. The pH values of these cultures cover a range of from 4.5 to 5.6 in the Witte peptone, and 4.6 to 5.8 in the synthetic medium.

Taking the three coli charts (III, IV and V) together, we find no qualitative differences in the results obtained with the three media or the two incubation periods, in so far as the methyl red test is concerned. This goes to show that incubation for three days at 30°C. is sufficient to give the characteristic methyl red reaction by coli strains from both fecal and non-fecal sources.

A careful analysis of these curves reveals several interesting facts. We find that the hydrogen ion concentration of the aerogenes cultures is generally higher in the synthetic than in the Witte peptone-glucose-phosphate medium either after a three or five days' incubation period. This would indicate that the production of alkalinity by aerogenes strains is more rapid in the Witte peptone medium than in the synthetic. In the former the peak of the H. I. C. curve for the three days' period is in the majority of cultures located at pH 6.3, while in the synthetic medium the peak lies at 5.7. A still more striking difference is shown in the five-day curves. In the synthetic medium the pH peak lies at 6.5, and the curve covers a range of only 6.0 to 6.8, whereas in the Witte peptone phosphate glucose medium the curve extends over a wider pH scale, 6.0 to 7.6, with the peak at 6.8.

In the Difco peptone medium the results are of peculiar interest. Setting aside for the present the few discrepancies (26 out of 447 aerogenes cultures which gave the methyl red positive test), a remarkable similarity between the Witte and the Difco peptone medium curves is observed. They almost parallel each other, from the range of pH 6.0 to 7.5. Their four peaks at pH 6.4, 6.6, 6.8 and 7.3, and the three troughs at 6.5, 6.7, and 7.1 correspond almost exactly.

REVERSION OF REACTION

The principle involved in the differentiation of the coli from the aerogenes type of bacteria by means of the Clark and Lubs media and the methyl red test is as follows. *B. coli* soon reaches a growth-inhibiting or lethal zone of hydrogen ion concentration and remains there; *B. aerogenes*, on the other hand, by virtue of its peculiar metabolic properties, continues its action upon the medium and progressively raises the pH value to a maximum which does not in itself retard further development of the organism. *B. coli* rapidly attacks the glucose, with relatively large acid production, while *B. aerogenes* produces more gas and correspondingly less acid during the early stages of growth than the coli type. *B. aerogenes* brings about a reversion of reaction which after prolonged incubation becomes more and more apparent.

This reversion of reaction in aerogenes cultures has led Kligler and a number of other investigators to assume that it is due to the production of ammonia from a peptone-glucose medium after exhaustion of the sugar. In their further study of the subject, Clark and Lubs disproved this view. They showed that reversion of reaction can take place in a synthetic medium free from peptone, and further proved that an extreme reversion can be obtained in a synthetic medium containing an ammonium salt as a source of nitrogen, although the content of total nitrogen is reduced to a point at which its participation in any form in changes of reaction of the medium would be insignificant. They come to the conclusion, therefore, that "An increase in ammonia may accompany the reversion of reaction, but the amount liberated is inadequate to account for the extent of the reversion." Under such conditions they believed that "It should not be assumed that the reversion is due solely to ammonia production."

These conclusions of Clark and Lubs do not give us a satisfactory explanation as to the cause of the reversion, nor do they lead us to think that they deny ammonia production in a glucose-peptone medium. It remained for Ayers and Rupp (1918) to furnish a plausible explanation for the phenomenon of reversion in a sugar-peptone medium. They demonstrated that there is

a simultaneous acid and alkaline fermentation in which the salts of organic acids produced from glucose are converted into carbonates or bi-carbonates, which in turn cause the reversion in reaction in a *B. aerogenes* culture.

In our attempts to follow up the work of Ayers and Rupp the Sörensen method of determining primary amino acid was employed, in addition to the ammonia determination method of Folin. It was hoped that some definite relationship between the formol titration and the ammonia figures could be established.

The Clark and Lubs peptone-phosphate-glucose medium was again made use of. Flasks containing 500 cc. of the medium were inoculated with a loopful (standard 4 mm. loop) of a twenty-four-hour culture of *B. aerogenes* grown in the same medium, and incubated at 30°C. At the end of 1, 2, 3, 5, and 10 day intervals the required amount of culture material was removed and subjected to the tests for sugar, amino acids, ammonia and hydrogen ion concentration. Eight representative strains of bacteria were selected for the test. Two were *B. coli* isolated from animal feces, and the remaining six aerogenes types from various apparently uncontaminated soils.

In the following table the Sörensen titration figures are recorded as the numbers of cubic centimeters of $\frac{N}{20}$ NaOH required to neutralize 100 cc. of the test medium. The ammonia figures are given as milligrams of ammonia per 100 cc. of the culture fluid.

The results show several interesting points. A close relationship appears to exist between the sugar utilization and the hydrogen ion concentration. In the aerogenes cultures the complete reversion of reaction took place within five days' incubation, and the sugar supply was practically exhausted in the same period. On the other hand, in the coli cultures the glucose was only partly fermented, and the hydrogen ion concentration remained constant after three days of incubation. No definite relationship between the Sörensen titration and the ammonia figures could be established. Irregularity of ammonia production is noticeable. One point stands out clearly from the rest; the amount of ammonia recorded in the *B. aerogenes* cultures was no greater, after ten days' incubation than in the control.

CULTURES		DAYS	pH	SÖRENSEN	AMMONIA	SUGAR
Control	Uninoculated		6.9	10.0	3.17	+
Aerogenes type	No. 34 Soil	1	5.2	10.0	2.20	+
		2	4.9	10.0	1.40	+
		3	4.9	20.0	1.70	+
		5	6.1	15.0	1.60	Trace
		10	6.9	10.0	2.10	Trace
	No. 37 Soil	1	5.4	20.0	2.75	+
		2	5.4	10.0	2.10	+
		3	6.1	10.0	1.92	Trace
		5	6.7	20.0	2.55	Trace
		10	6.8	15.0	2.01	Trace
	No. 24 Soil	1	5.9	10.0	1.40	+
		2	5.9	20.0	1.84	+
		3	6.3	15.0	2.50	Trace
		5	6.8	10.0	3.10	0
		10	6.8	10.0	2.54	Trace
	No. 44 Soil	1	5.9	15.0	1.45	+
		2	5.9	20.0	1.75	+
		3	6.3	10.0	2.04	0
		5	6.4	10.0	1.70	0
		10	6.5	35.0	2.80	0
	No. 20 Soil	1	5.9	20.0	1.50	+
		2	5.9	30.0	1.45	+
		3	6.3	20.0	1.78	Trace
		5	6.4	10.0	2.80	Trace
		10	6.8	20.0	3.02	Trace
	No. 21 Soil	1	5.1	20.0	1.64	+
		2	5.0	30.0	2.90	Trace
		3	5.3	10.0	3.10	Trace
		5	6.0	20.0	2.45	Trace
		10	6.5	30.0	2.70	0
Coli type	No. 5 Human feces	1	4.9	15.0	2.40	+
		2	4.6	20.0	3.30	+
		3	4.8	10.0	2.10	+
		5	4.7	10.0	3.10	+
		10	4.7	20.0	3.04	+
	No. 27 Human feces	1	4.8	10.0	1.40	+
		2	4.6	10.0	1.50	+
		3	4.7	15.0	2.90	+
		5	4.7	20.0	2.10	+
		10	4.7	15.0	3.12	+

The interpretation of the results is fraught with some difficulty. Clark and Lubs, and Ayers and Rupp did not deny that ammonia production may accompany the phenomenon of reversion. Kendall's theory of ammonia recession may in part explain a certain phase of reversion, and the claim of Rettger and Berman, and others, that ammonia is a readily available food for bacteria, and that it is not an end product, but an intermediary stage of metabolism, may throw considerable light on the subject.

GAS PRODUCTION

Escherich (1885) clearly demonstrated the production of gas in glucose and lactose media by his two types of intestinal organisms. Arloing confirmed the observation of Escherich. It was not until after the appearance of the fermentation tube (1890–93) of Theobald Smith, however, that the determination of gas volume and ratio formed part of our scheme of differentiation of bacteria. For almost twenty years this method reigned supreme and its accuracy as a quantitative procedure was little questioned.

Although Harden, Walpole and Thompson had improved the Smith method, the analytical error was not eliminated. Keyes (1909) introduced the vacuum method of exact gas analysis, and determined the gas ratio of *B. coli*. His work was followed by that of Rogers and his associates (1914–15), who firmly established the gas ratio of *B. coli* and *B. aerogenes*, namely approximately 1:1 (CO_2: H) for the former, and 2:1 for the aerogenes type. Since then there appears to be no doubt that the principle of gas-ratio as a differential test is well founded.

In the present study the gas production was at first observed in the Durham fermentation tube. A gas volume of at least 10 per cent was recorded as a positive test for gas, while the acid reaction in the same tubes was roughly determined with Bromcresol-purple, a distinct yellow color being recorded as a positive reaction. In the use of the Durham fermentation tube a long inner tube (3 inches) was employed to facilitate the reading of the gas volume, which was done by the Frost gasometer. Al-

though this method is very crude, some interesting data were obtained.

Cultures of the *B. coli* type were seldom observed to attain a gas volume greater than 40 per cent when the medium had 1 per cent of glucose, the amount of gas usually being between 20 and 40 per cent. More specifically, of 173 fecal strains of *B. coli* cultures used only 8 gave a gas volume of 40 per cent, all of the remaining 165 falling below this figure. On the other hand, 322 out of 447 aerogenes strains gave a percentage higher than 40, 74 produced 40, and only 51 gave less than 40 per cent.

On account of the lack of proper facilities gas determination by the exact method was not undertaken until toward the end of the present study. Considerable time was spent in designing a vacuum pump which was a modification of the Sprengel pump, the plan of the Boltwood pump being followed to a large extent. As the Boltwood pump is not adapted for the collection of gases, and as no provision is made for removing the minute air bubbles which collect in the mercury, it had to be further modified. The distinct features of the new design of vacuum pump are: (1) It can be employed for exhaustion of the gases as well as their collection, (2) It possesses a device for the removal of air bubbles from the mercury, and (3) Its operation is automatic and continuous. As it is planned to give a complete description of this apparatus in a separate publication (Chen), further discussion of its design and application is omitted here, except a brief allusion to the following table which gives the representative results of one of the few estimations which were hurriedly made at the close of the present study, and which as far as it goes confirms the gas ratio of Rogers, Clark, etc.

Gas-production in vacuum bulbs

TYPE	SOURCE	TOTAL GAS	RATIO			
			CO_2	H_2	CO_2/H_2	Residue
		cc.				
Aerogenes.....	Soil no. 7	20.80	7.83	3.62	2.16	9.35
Coli.....	Human feces No. 36	11.70	4.26	3.90	1.06	3.54

THE VOGES AND PROSKAUER REACTION

This reaction was first observed by Voges and Proskauer (1898) in their studies on the bacteria of hemorrhagic septicemia. It was soon found that certain members of the colon group gave the reaction, and that on this as a basis the typical and atypical, or the fecal and non-fecal, types coming under this group could be differentiated from each other. Durham (1901), McConkey (1905–09), Archibald (1907), Bergey and Deehan (1908), Ferreira, Horta and Paredes (1908), West (1909), Copeland and Hoover (1911), Clemesha (1912), Kligler (1914), Levine (1916), and more recently Hulton, Greenfield, Johnson, Winslow and Kligler, Burton and Rettger, Winslow and Cohen, and Rogers and his associates have made use of this reaction. As a result the test has been given added significance, and promises to be one of the most important methods of differentiating the coli from the aerogenes type of organisms.

The chemistry of this reaction was not known until the publication of the thorough and painstaking researches of Harden and his co-workers. Harden (1905) in his study of the fermentation of glucose by colon bacilli discovered two important facts concerning the end products of glucose fermentation of the coli and aerogenes types of bacteria. He found that *B. coli* gives a high acidity, with only a partial utilization of the glucose, whereas the aerogenes type produces low acidity and complete exhaustion of the sugar. He concluded that "*B. lactis aerogenes* acts upon glucose in a totally different manner from *B. coli* and is therefore to be regarded as a distinct organism."

Harden and Walpole (1905–06) recognized a new substance, glycol, among the usual products of glucose fermentation, such as lactic, acetic, formic and succinic acids, alcohol and carbon dioxide. According to them, this new substance, crude glycol, contains a large amount of 2:3 butylene glycol (CH_3-CHOH-CHOH-CH_3) which in the presence of atmospheric oxygen is oxidized to acetyl-methyl-carbinol (CH_2-CO-CHOH-CH_3). This volatile reducing substance is further oxidized in a relatively strong alkaline solution to di-acetyl (CH_3CO-CO-CH_3). The

diacetyl is colorless as such, but when brought into contact with a small amount of peptone or other protein material produces the characteristic eosin-like red coloration.

Walpole (1910) further observed that by passing a current of air through the *B. aerogenes* culture five to six times as much acetyl-methyl-carbinol is produced as when the culture fluid is left undisturbed. Thompson (1911) found that the chemical action of *B. cloacae* of Jordan on glucose is practically the same as that of *B. aerogenes*, in so far as the production of carbinol is concerned.

The actual correlation of the Voges and Proskauer reaction with the source of the organism and with its other characteristics was the work of Levine (1916). Soon after Rogers and his coworkers announced the definite relationship between the gas ratio, gas volume and the hydrogen ion concentration of the coli and aerogenes types of bacteria, Levine showed that an added correlation could be established with the Voges and Proskauer reaction. He found that bacteria of the aerogenes type consistently gave a positive V and P reaction, whereas those which were positive to the methyl red test were regularly V and P negative.

In the present study an attempt was made to determine: first, the relative value of the three media previously described in this paper, namely the Clark and Lubs' Witte peptone-glucose-phosphate, the synthetic phthalate-glucose-phosphate, and the Difco peptone-glucose-phosphate medium, for the study of the Voges and Proskauer reaction; second, the most favorable period of incubation; and third, a rapid and practical method for carrying out the test.

In the original method of applying the Voges and Proskauer test the alkaline fluid was exposed to the atmosphere for twenty-four hours or longer at room temperature. Walpole tried to hasten the oxidation process by passing a current of air through the medium, while West, following "Test No. 1" of Revas, boiled his cultures. More recently Levine introduced various oxidizing agents, and Bunker, Tucker and Green (1918) advocated the use of Syracuse watch glasses. These methods, and particularly the last two, appear to us too uncertain and too laborious.

The technique finally adopted in this work was as follows: At the end of one, three and five days' incubation periods 5 to 6 cc. of the culture fluid were well shaken in the test tube with an equal amount of 10 per cent potassium hydroxide solution. The tubes were placed in an incubator having a constant temperature of 30°C. At the end of one to three hours the tubes were again vigorously shaken until the liquid became foamy. As a rule, the eosin-like coloration made its appearance quickly, and the color deepened throughout the tube, instead of becoming visible as a ring or a surface layer. Without an exception, a decidedly positive Voges and Proskauer reaction (maximum color production) could be observed within a few hours (two to three) by this method. West used a more or less similar shake method ten years ago. He said: "After heating the tube, shake well, or blow through it to bring the red color out."

By the use of the method just described a total of 3725 individual Voges and Proskauer tests were made in the three different glucose-phosphate media, following three different incubation periods. The results are shown in the following chart (VI).

The chart shows clearly that there was no difference in the color reaction as the result of differences in the length of the incubation periods, one, three and five days, or of the different brands of peptone employed. Although the coloration in the synthetic medium was not so uniformly strong as in the peptone media, the reaction was always sufficiently pronounced to be called distinctly positive

Different strains manifested a striking difference in the degree of coloration which they gave. Not a single aerogenes culture, however, failed to give the reaction. On the other hand, not one of the colon strains, either from soil or feces, responded to the test.

An attempt was made further to determine the minimum incubation period in which color production can be obtained. A small collection of typical and representative strains of *B. aerogenes* was tested in the same three media after four, ten and fourteen hours of incubation of the cultures at 30°C. The results showed that a positive Voges and Proskauer reaction may be ob-

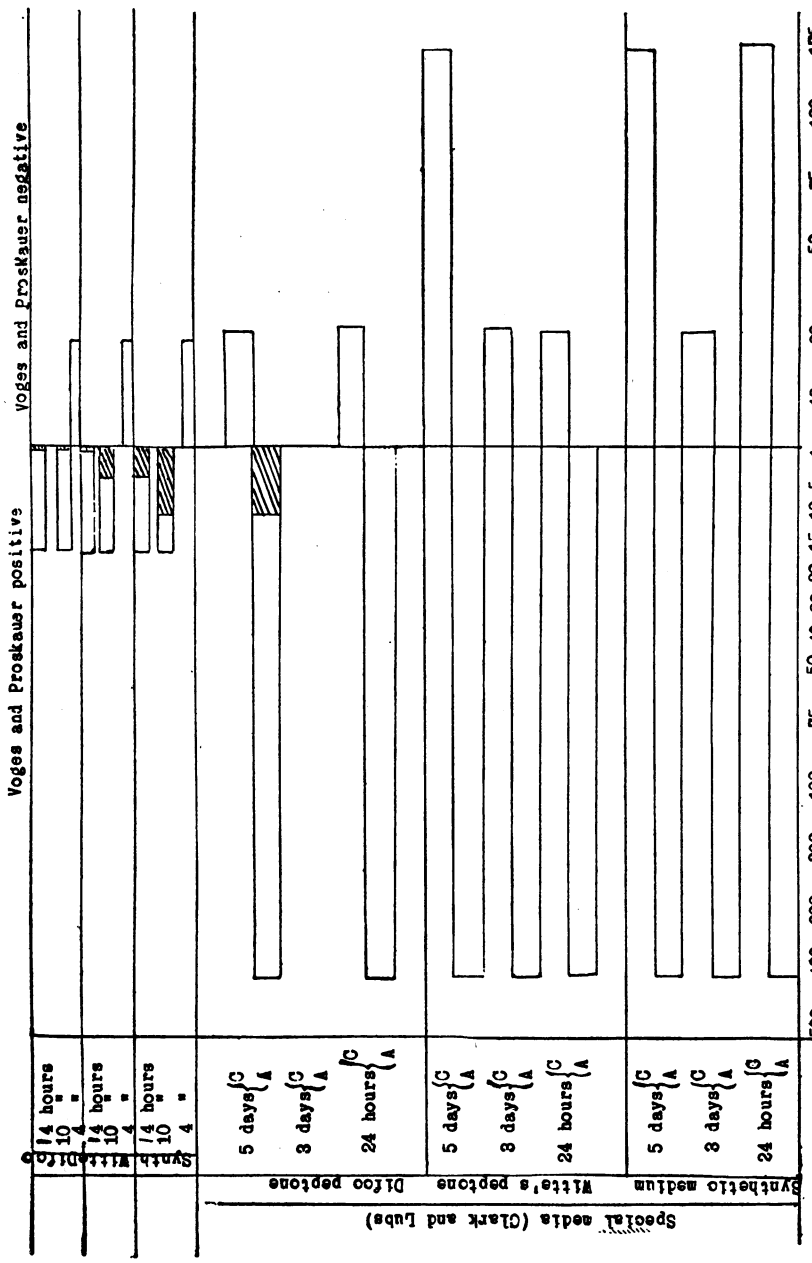

CHART VI. Upper section, showing Voges and Proskauer reaction in very young cultures of B. aerogenes. Lower, main section, showing V. & P. reactions in the different media after one, three and five days' incubation at 30°C.
C = Coli type; A = aerogenes type. The figures below indicate number of strains. Shaded areas show weak reactions.

tained in cultures which are only ten to fourteen hours old (chart VI, upper part).

THE DEPORTMENT OF MIXED CULTURES (COLI AND AEROGENES TYPES) TOWARD THE METHYL RED TEST AND THE VOGES AND PROSKAUER REACTION

One strain of typical *B. coli* of fecal origin was grown in symbiosis with 56 different strains of *B. aerogenes* obtained from soil. Each of the strains had been cultured separately in plain broth for twenty-four hours, and a loopful of each tube was inoculated into the Witte peptone- and the synthetic medium of Clark and Lubs. The mixed cultures were incubated for five days, one set at 24° and a second at 37°C. The hydrogen ion concentration was then determined with methyl red as indicator, and the Voges and Proskauer test was made as described earlier in this paper.

The results of the study are given in the accompanying chart (VII). They show that in the peptone-phosphate-glucose medium, both at 24° and at 37°, the hydrogen ion concentration of the aerogenes type of organism was partly disturbed, and that in the synthetic medium the acidity of the colon type almost entirely obscured that of *B. aerogenes*. The Voges and Proskauer reaction as ordinarily given by the aerogenes type was not at all affected in the synthetic medium at 24°, and only one culture in the peptone medium failed to give the characteristic reaction when grown at 24°C. Of the 56 tubes incubated at 37°, 14 were negative by the V and P test, 4 in the synthetic and 10 in the peptone medium.

Briefly stated, when both types of organisms are present in any given culture the methyl red test reveals the presence of the colon type, while the aerogenes type responds to the Voges and Proskauer test when the temperature of incubation has been as low as 24°. At the higher temperature only a small number of the cultures are negative to the V & P test. The permanency of the Voges and Proskauer reaction is here clearly demonstrated.

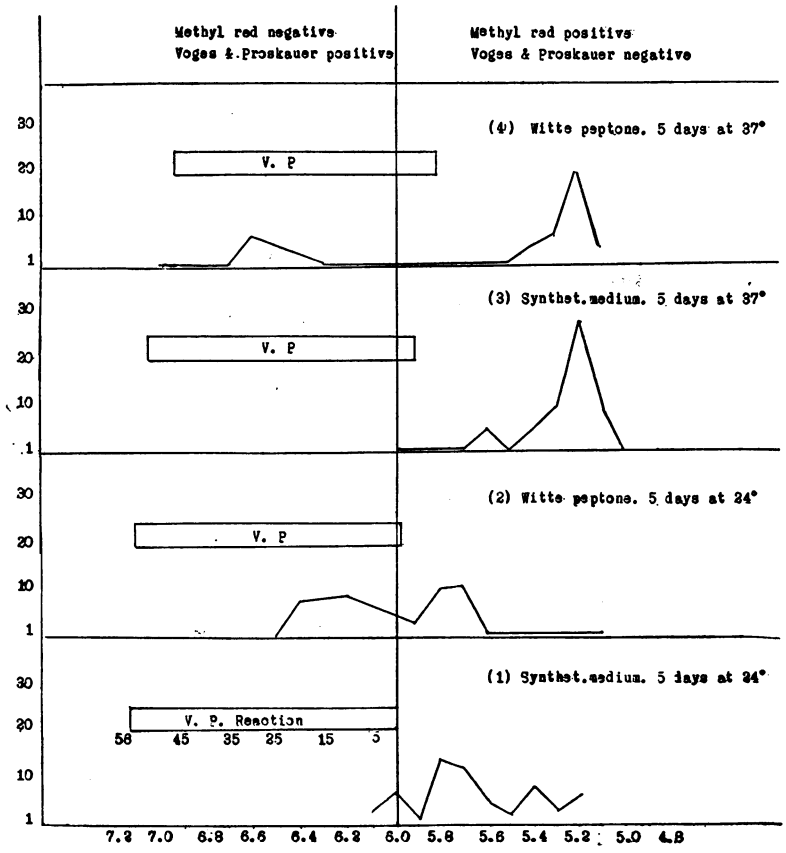

CHART VII. SHOWING SYMBIOTIC REACTIONS OF ONE STRAIN OF B. COLI WITH 56 STRAINS OF B. AEROGENES

DIFFERENCES IN NITROGEN UTILIZATION AS A BASIS FOR DISTINGUISHING B. COLI FROM B. AEROGENES. THE URIC ACID TEST OF KOSER

The selective action of bacteria on carbohydrates has long been recognized, and on this principle many tests have been devised, and different types of bacteria have been successfully separated. The differentiation rests, however, on the character of the end products of carbohydrate fermentation, as for example

the formation of acid and gas, and the production of carbinol and diacetyl.

Much attention has also been given in recent years to the differentiation of bacteria on the basis of nitrogen utilization, in the form of ammonia, amino acids, di-amines, nitrates, etc. For example, it was shown by Uschinsky that *B. coli* can attack amino acids like asparagine in a purely synthetic medium, whereas *B. typhosus* is as a rule unable to utilize it as a source of nitrogen. Quite recently Koser (1918 and 1919) succeeded in a somewhat similar manner in differentiating between the *B. coli* and *B. aerogenes* types of organisms.

In his extensive study of nitrogen utilization by bacteria in media of definite chemical composition, Koser observed that *B. aerogenes* readily attacks uric acid or hypoxanthine as the only source of nitrogen, while *B. coli* is void of this property. In other words, he found that the aerogenes type possesses the unique power of seizing upon the nitrogen of the purin ring, whereas the coli type leaves this portion of the uric acid molecule intact, and fails in its development unless some other nitrogenous substance which furnishes available nitrogen is present.

In the present investigation all of the strains isolated from feces and soils were employed in a further study of this phase of bacterial nutrition. They were inoculated into the uric acid synthetic medium of Koser. In a large series of tests other purin bases were employed in the place of the uric acid, the basic composition of the synthetic medium being the same as when the uric acid was used. These substances were xanthine, caffein and theobromin. The uric acid synthetic medium as prepared by Koser has the following composition:

Distilled ammonia-free water	1000.0 cc.
NaCl	5.0 grams
$MgSO_4$	0.2 gram
$CaCl_2$	0.1 gram
K_2HPO_4	0.1 gram
Glycerol	30.0 grams
Uric acid	0.5 gram

The medium was tubed in 5 cc. portions and sterilized for fifteen minutes at 12 pounds extra pressure. The test tubes

which are used must be scrupulously clean; small amounts of nitrogenous matter left on the walls may furnish sufficient nitrogen for the organisms to multiply so as to obscure the sharp distinction between the appearance of the *B. coli* and *B. aerogenes* tubes after the required time of incubation, four to five days at 30°C.

A twenty-four hours growth of the organism under observation was used as a rule as inoculum, and this was transferred with precaution, so as to limit as far as possible the amount of cell material and products of bacterial metabolism which are carried over. The results of the tests with the uric acid medium are summarized in the following table.

Table showing results of uric acid tests

	POSITIVE	NEGATIVE	TOTAL
Aerogenes strains from soils.....	447	0	447
Coli strains from feces.....	0	173	173
Coli strains from soils.....	10	10	20

All of the aerogenes strains (soil) gave a very pronounced growth in the uric acid medium, while none of the fecal strains of the coli type clouded the medium or in any way showed indications of development. The differentiation was in every instance sharp though the degree of turbidity varied much. However, considerable discrepancy was observed among the coli strains isolated from soils. Of the 20 cultures employed 10 were uric acid positive and 10 negative. In the tubes in which the uric acid was attacked the growth was as luxuriant as in typical cultures of the aerogenes type. Lack of correlation here of the 10 uric acid positive strains of soil coli with the methyl red test and Voges and Proskauer reaction may be of some significance as pointing to the possibility that these ten strains are of a type intermediate between the coli and the aerogenes.

The same results were obtained in the xanthine synthetic medium as in the uric acid, except with the soil strains of *B. coli*, which goes to show further that the failure of *B. coli* to utilize the nitrogen is due to its inability to disrupt the purin ring,

which contains the nitrogen. All of the soil coli strains failed to attack the xanthine, and hence reacted like typical *B. coli.* Further investigation may show that xanthine may be superior even to uric acid for differentiating these two types of bacteria, unless types intermediary between coli and aerogenes exist which in uric acid medium are sharply distinguished. With two exceptions, both types of bacteria failed to develop in the caffeine and the theobromine medium. The results with the xanthine, caffeine and theobromine media are shown in the following table.

Table giving results with xanthine, caffeine and theobromine media

ORGANISMS	XANTHINE		THEOBROMINE		CAFFEINE		TOTAL
	+	−	+	−	+	−	
Aerogenes (soils)	55	0	0	55	0	55	55
Coli (soils)	0	20	0	20	0	20	20
Coli (feces)	0	55	2	53	0	55	55
Aerogenes (Am. Mus. Nat. Hist.)	10	0	0	10	0	10	10
B. communior (Am. Mus. Nat. Hist.)	0	5	0	5	0	5	5
B. communis (Am. Mus. Nat. Hist.)	0	5	0	5	0	5	5
Aerogenes (Rogers)	6	0	0	6	0	6	6
Aerogenes (Winslow and Cohen)	5	0	0	5	0	5	5
Total							161

FERMENTATION OF GLUCOSE, LACTOSE, SUCROSE, ADONITOL AND DULCITOL

It was at first intended to classify the present collection of strains according to McConkey's primary divisions, on the basis of fermentation of glucose, lactose, sucrose and dulcitol. Adonitol was also employed because of the claims of Rogers and others that the so-called fecal and non-fecal types of *B. aerogenes* can be differentiated in this way. Owing to our inability to obtain sufficient dulcitol the classification study with McConkey's four sugars was incomplete. The results are presented in the following table.

BACTERIAL STRAINS	GLUCOSE		LACTOSE		SUCROSE		ADONITOL		DULCITOL		TOTAL
	+	−	+	−	+	−	+	−	+	−	
Aerogenes type (from soils)	447	0	447	0	338	109	152	295			447
Coli type (from soils).....	20	0	20	0	15	5	5	15			20
Coli type (from feces).....	173	0	173	0	91	82	30	143			173

Results with dulcitol too incomplete to include in table. The figures indicate both acid and gas production.

All of the strains fermented glucose and lactose. Sucrose was attacked by both the colon and aerogenes types, although the high gas ratio group was as a group more active than the other, excluding the soil coli.

The present results do not bear out the contention that the fecal and non-fecal types of aerogenes may be differentiated by fermentation in adonitol, as 152 soil aerogenes strains out of the 447 attacked the adonitol, whereas the remaining 295 did not. These findings agree in principle with those of Winslow and Cohen, who observed that "a greater proportion of *B. aerogenes* from the unpolluted sources attacked adonite than did those from the polluted waters."

ATYPICAL STRAINS

In the present collection of organisms which resemble the aerogenes type, 18 at first appeared to occupy an intermediate position where complete correlation could not be established. They persisted in giving methyl red positive and Voges and Proskauer positive reactions in all three of the media employed, after one, three and five days' incubation. Impurity of the cultures was suspected and repeated platings were resorted to. In this way the number of non-correlating organisms was reduced from 18 to 4, although contaminating organisms could not be demonstrated.

The methyl red positive strains whose hydrogen ion concentration was on the border line of the methyl red range (5.7 to 5.9) were made to return to their typical methyl red negative reactions by repeated plating, their pH values being raised to

6.2–6.5. This would indicate that variation within certain limits in the hydrogen ion concentration is to be expected. After 39 replatings the above-mentioned 4 strains still failed to correlate, however, in so far as the methyl red test and the Voges and Proskauer reactions are concerned, both being positive. It is unfortunate that we were unable at the time to make exact gas determinations on these 4 strains, since Clark and Lubs have claimed that "when a perfectly clear-cut correlation fails, there is generally found some abnormality in gas-production. . . ."

All of the 4 peculiar strains gave a pronounced Voges and Proskauer test and were able to attack the purin ring in uric acid; hence, in so far as these two reactions are concerned these organisms resembled typical *B. aerogenes*.

Rogers and his associates reported that the majority of their atypical strains gave a pH value ranging from 5.6 to 6.0, and that most of them (11 out of 16) were Voges and Proskauer positive. Repeated replating might have materially reduced their number of atypical strains, as the pH values given by them were so near the border line of the methyl red indicator range, and as a small variation in the hydrogen ion concentration must be expected of all bacteria.

CORRELATION OF CHARACTERS

Chart VIII presents graphically the results obtained in the study of the various characters of the 447 aerogenes and 173 fecal strains of coliform bacteria. The tests embraced the methyl red, uric acid and Voges and Proskauer reactions, deportment towards glucose, lactose, sucrose and adonitol, indol formation and gelatin liquefaction. As a matter of brevity, the results obtained with the 20 coli strains from soil and the 4 atypical strains are not presented here in chart form. They differ little from the above, aside from a difference in indol production and adonitol fermentation, and the points already mentioned with reference to the methyl red, uric acid and Voges and Proskauer.

It will be observed in the accompanying chart that perfect correlation, exists for both the aerogenes (447) and the fecal coli

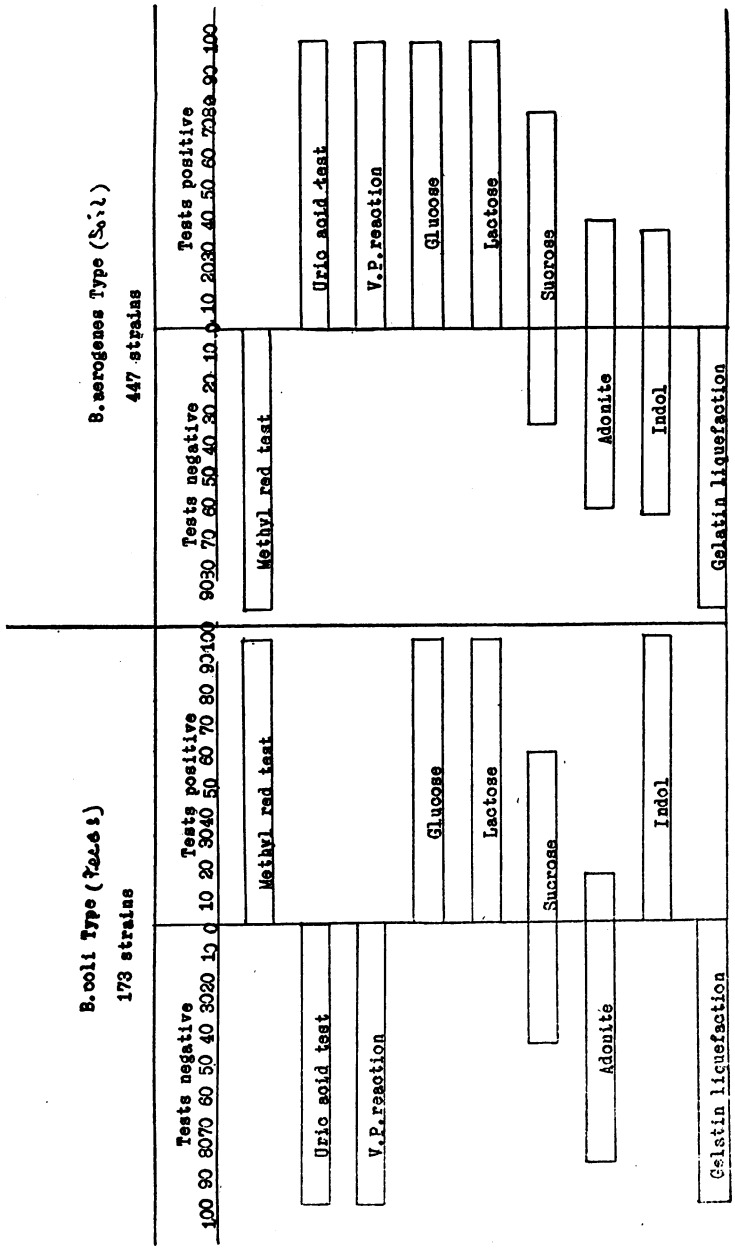

CHART VIII. CORRELATION OF CHARACTERS OF B. COLI AND B. AEROGENES TYPES

(173) types, aside from the reactions in sucrose and adonitol, and indol production of the aerogenes type.

A complete study of gas production in the Durham fermentation tube was made of all of these organisms, both as to volume and gas ratio, in so far as the method would permit, 40 per cent being the arbitrary limit placed for the coli type, and above 40 per cent being taken as an indication of the aerogenes type. Though very crude, this method permitted us to assume from the results obtained a complete correlation between gas volume, gas ratio, the methyl red, uric acid and the V and P reactions. However, on account of the very faulty method employed in the quantitative study of gas production these results are not included in the charts or in any of the tables.

GENERAL DISCUSSION

A survey of the present investigation leads to the conclusion that there exist in nature two distinct types within the colon group of bacteria. These two types, now generally designated as *B. coli* and *B. aerogenes*, can conveniently be set apart from each other by the newer tests.

The direct plating method is better adapted for the isolation of the soil organisms than the combined preliminary enrichment and subsequent plating procedure, because it permits of the study of numerical relationships between bacterial types and their habitat and of the determination of the relative numbers of each type.

The failure to isolate *B. aerogenes* from the feces of man and animals in the present study should not be taken as evidence that this organism is absent from the intestine, but rather as a result of circumstances which permitted its being overlooked. This apparent absence or scarcity is not at all surprising, as Rogers and his associates (1914) found only one high ratio strain in 150 coli-like organisms isolated from bovine feces.

Nothing of importance could be gained through the morphological study of this group of bacteria, since both types present practically the same microscopic picture. The indol test is of

little value, also, and should be used with caution. While all of the *B. coli* strains produce indol, the percentage of positive tests with the aerogenes type is too large to make the test a practical one. The Ehrlich method of determining indol formation is decidedly more delicate than the Salkowski sulphuric acid test, and must be regarded as the more reliable. In the present investigation gelatin liquefaction has been of no value as a test.

The constancy of the hydrogen ion concentration in cultures of the *B. coli* type, as determined by the colorimetric method, is proved beyond doubt by the present study. The hydrogen ion concentration can be adequately measured for practical purposes by the methyl red indicator, as Clark and Lubs have claimed. The synthetic medium of Clark and Lubs appears to be best suited for the colorimetric determination of acidity. Witte's peptone-phosphate-glucose medium of Clark and Lubs answers the purpose well for which it is intended provided that it is kept colorless or practically free from color during the process of sterilization.

Owing to the interference of simultaneous alkali production the limiting hydrogen ion concentration of the aerogenes strains could not be so easily determined. The results of the present investigation show that the hydrogen ion concentration of these strains is completely masked after five days' incubation both in the synthetic and in the Witte peptone-phosphate-glucose medium.

A new set of experiments was conducted to determine daily the hydrogen ion concentration of a collection of 60 aerogenes strains in the synthetic medium for a period of three weeks, in order to ascertain the degree of OH-ion concentration reached by the cultures. The H-ion concentration began to decrease at the end of three days of incubation, and the pH value of the OH-ion concentration proceeded to increase steadily and progressively until the end of the experiment. The greatest H-ion concentration was 4.7, and the highest OH 7.4. It must be apparent, therefore, that length of incubation and temperature are two very important factors in the interpretation of results. If the colorimetric determination is made too soon, or the incu-

bation temperature too low, colorimetric determinations with the methyl red indicator may be positive and correlation with the other tests will be lacking.

Difco peptone-phosphate-glucose medium is less well adapted for the study of hydrogen ion concentration of the aerogenes type than the synthetic and the Witte peptone media of Clark and Lubs, and a greater proportion of failures to correlate must be expected, expecially if the exacting conditions as to length of incubation and incubation temperature are not supplied. The difference appears, however, to be chiefly one of degree rather than kind.

The Voges and Proskauer reaction has proven itself in the present investigation to be of very great value in differentiating between the coli and aerogenes types of bacteria. Neither the character of the medium nor the period of incubation seems to interfere with the carbinol formation. A colorless medium is as important, however, for this test as for the colorimetric determination of the hydrogen ion concentration, owing to the fact that, while the characteristic coloration of the tubes cannot be mistaken, it may be at times obscured by coloring matter in the medium itself, especially if the reaction is relatively weak. Furthermore, intelligent execution of the test is absolutely necessary.

The Voges and Proskauer reaction should be observed within shorter incubation periods than have been customary. A weak reaction persists only for a short time, while a strong reaction may last for several days, with a gradual fading away of the color. A period of from two to eight hours may be regarded as sufficient for a positive V and P reaction, if correctly carried out. Abundant oxygenation of the medium after the addition of the alkali, and proper incubation are important factors.

The uric acid test of Koser constitutes another reliable correlation test. Great care must be exercised, however, in the employment of test tubes, etc. which are free from adhering nitrogenous matter. Koser obtained excellent results with uric acid and hypoxanthine. In the present work xanthine, theobromine and caffeine were employed besides uric acid, the xanthine and uric acid giving very gratifying results. No growth could

be obtained, however, in the theobromine and caffeine synthetic media, either of the coli or of the aerogenes type. This was due, in all probability, to an inhibitive or antiseptic action of these substances, even in the small amount in which they were used. *B. aerogenes* is able to attack the purin ring of xanthine, hypoxanthine and uric acid, and thus appropriate nitrogen in a sufficient degree to develop in the synthetic medium more or less luxuriantly, whereas *B. coli* lacks this property. The differentiation is sharp.

SUMMARY

The present study of coli-like organism in soils of known sanitary quality has shown the great predominance of the aerogenes-cloacae type. Of 467 strains of bacteria isolated from various soils 430 were identified as *B. aerogenes*, 17 as *B. cloacae*, and only 20 as *B. coli*. Furthermore, the sources of the coli strains were shown by the sanitary survey to be not entirely free from animal pollution. All of the 173 organisms found in the feces of 7 men, 2 monkeys and 14 domestic animals were typical *B. coli*. It is apparent from these observations that there is a definite correlation between these types of bacteria and their origin.

An almost perfect correlation could be established by the methyl red, Voges and Proskauer and the uric acid tests.

The limiting hydrogen ion concentration of the coli cultures, as determined by the colorimetric method, varied from pH 4.5 to 5.6 in the synthetic medium, and from 4.6 to 5.8 in the Witte peptone-phosphate-glucose medium. The final hydrogen ion concentration of the cloacae-aerogenes type could not be accurately determined on account of the simultaneous acid and alkali production. The pH value obtained under similar conditions ranged from 6.0 to 7.4 in the Witte peptone medium, and from 6.0 to 6.8 in the synthetic medium.

The respective hydrogen ion concentrations of the colon and aerogenes types of bacteria may be adequately determined for practical purposes by methyl red as an indicator, provided the neutral tint reactions are compared with the reactions obtained by brom cresol purple or some other sharp indicator as a check.

The Voges and Proskauer method of distinguishing between *B. coli* and *B. aerogenes* has proven even more satisfactory than the methyl red test, in that it is simple in operation, and when correctly carried out thoroughly constant in its results. When used with precaution, the uric acid test also is of fundamental importance in differentiating fecal coli from the soil aerogenes type of bacteria.

No definite correlation could be established by means of the indol test. Neither did motility study prove to be of practical value.

Adonitol fermentation did not prove itself to be a satisfactory method of differentiating fecal from non-fecal strains of *B. aerogenes*.

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