CORRELATION OF CITRATE UTILIZATION BY MEMBERS OF THE COLON-AEROGENES GROUP WITH OTHER DIFFERENTIAL CHARACTERISTICS AND WITH HABITAT.

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In a previous study of the utilization of salts of various organic acids it was found that two sections of the colon-aerogenes group were clearly distinguished by the use of a chemically definite medium containing sodium, potassium, or ammonium citrate as the only source of carbon (Koser, 1923). The methyl red positive, Voges-Proskauer negative cultures of fecal origin —typical *Bact. coli*—were incapable of utilizing the citrate radical as a source of carbon and consequently did not develop, while the methyl red negative, Voges-Proskauer positive cultures—*Bact. aerogenes* and allies —readily attacked the citrates. Throughout the previous investigation the testing of a number of different organic acids necessitated the employment of a limited number of colon-aerogenes strains. In view of the different habitats in which members of the colon-aerogenes group have been found in nature and of the recent interest attached to these types from a sanitary standpoint, it was deemed advisable to continue the study of citrate utilization by employing a larger number of strains from as many different sources as possible.

In the present investigation the ability to utilize the citrate radical has been compared with the source of the cultures in nature, with glucose metabolism in a buffered peptone glucose medium, as brought out by the well-known methyl red and Voges-Proskauer tests, and with the ability to utilize the nitro-

gen in the purin ring of uric acid. All non-spore-forming, Gram-negative rods fermenting glucose and lactose with the production of acid and gas and capable of growing aerobically were considered as members of the colon-aerogenes group. All of the strains used in the present study were isolated by the writer and consequently the history, including the source of original isolation, was definitely known. The cultures were obtained from locations which represented the opposite extremes of sanitary standards. One hundred and eighteen cultures were obtained from fecal specimens, while seventy-two cultures were isolated from soils as far removed from any chance of pollution as possible.

The fecal cultures were secured from man as well as from a number of domestic and laboratory animals. The method of isolation consisted in emulsifying part of the fecal specimen in sterile water and streaking a loopful of the resultant thick suspension over Endo plates. After eighteen to twenty-four hours incubation several colon-like colonies, never more than four or five from one specimen, were transferred to lactose broth and to plain agar slants. If the cultural properties in these media showed the organisms to belong to the colon-aerogenes group the process of fishing from well-isolated colonies on an Endo plate was repeated before the cultures were added to the stock collection. As a rule, only typical, flat, dark-red colonies with a metallic luster appeared on Endo plates streaked from the fecal specimens. Occasionally, however, in addition to this type of colony, lactose fermenters were found which produced raised pink colonies, frequently more or less mucoid. Whenever these appeared, one or two representative colonies, depending upon the proportion present on the plate, were also isolated as described and added to the stock collection for future tests. A few of the fecal specimens from laboratory animals, notably those from guinea-pigs, contained so few colon organisms that no colonies were obtained by direct streaking of emulsions on Endo plates. In these cases the fecal samples were added to lactose broth and after twenty-four hours incubation the resultant cultures were streaked on Endo plates. Representa-

tives of the colon group were usually obtained after this enrichment. After such a procedure, however, only one colony was picked as representative of each fecal specimen, unless more than one type of colon-like colony appeared on the plates.

Every effort was made to secure soil samples from locations where the chance of human pollution would be very slight. Most of the samples were collected in Virginia and Maryland within a radius of twenty miles of the District of Columbia and were taken from wooded hilltops, usually where the underbrush was very dense and where the possibility of any chance human pollution seemed to be very improbable. A few soil samples were taken from the South Mountain range of the Alleghanies in eastern Pennsylvania. These specimens were collected on mountain summits at a considerable distance from roads or trails. The nearest habitations, several small farmhouses, were a mile or more distant in a valley. While the possibility of pollution by small wild animals or birds could not be entirely excluded from any of these locations, nevertheless, it is believed that organisms obtained from such sources may justly be regarded as representative of a normal unpolluted habitat.

The procedure followed when collecting the soil samples consisted in first scraping away the surface layer of decaying leaves, etc., and then taking the sample from the surface soil, or from a depth of not more than 1 to 2 inches below the surface. A flat sterile spatula or other flat instrument was used for transferring the soil to sterile, wide-mouth glass bottles which were used as containers for transporting the specimens to the laboratory. The glass stoppers were protected by a layer of cotton covered with several thicknesses of gauze.

The isolation of colon-aerogenes organisms from soil was first attempted by preparing dilutions of the soil samples and plating directly from them. Owing to the scarcity of these types in the soils studied, however, this method failed to detect many organisms which were found upon the enrichment of larger portions of the samples in flasks of lactose broth. The method of isolation finally adopted was to add 10 to 15 grams of each soil sample to 75 cc. of 0.5 per cent lactose broth con-

tained in 200 cc. Erlenmeyer flasks. These were incubated at 30°C. and from the resultant cultures Endo plates were streaked after twenty-four hours incubation and sometimes again after four or five days.

The necessity for repeated examination of the soil cultures and of the Endo plates made from them was brought out by the occasional finding of colon group organisms which exhibited a delayed fermentation of lactose. While such organisms would be overlooked by the ordinary methods of examination, it was felt that they should be included in any study of the colon-aerogenes group and consequently the usual procedures were modified to a certain extent in order to detect them. When fishing colonies from Endo plates the selection was not limited to the usual red or pink colon colonies, but all forms were included which in any way resembled the type of colony formed by the colon group, whether showing the red coloration or not. These were transferred to 0.5 per cent lactose broth tubes and held for fourteen days or more at 30°C. All cultures thus found to be capable of fermenting lactose were streaked again over Endo plates and reisolated before adding them to the stock collection. By following this procedure several colon group cultures which exhibited a delayed fermentation of lactose were obtained. These cultures formed small colorless colonies on Endo plates after twenty-four hours at 30°C., but when held for several days the colonies became much larger, frequently assuming a deep pink mucoid appearance. When transferred to lactose broth there was no evidence of fermentation until after three or four days or occasionally longer. The hydrogen-ion concentration attained by these cultures in lactose broth was never as great as that of the fecal coli strains, but on the other hand the evolution of gas was very pronounced after the fermentation had begun. While members of the colon group which attack lactose tardily have seldom been reported, owing no doubt to the methods of examination employed, it is evident that they constitute an appreciable part of the flora of the soils studied in the present investigation, for of the 72 colon group cultures from soil, 14 exhibited a delayed lactose fermentation. Most

of these produced acid and gas in a 0.5 per cent lactose broth within three or four days at 30°C. although a few showed no evidence of fermentation until the seventh or eighth day. Colon group organisms showing a somewhat similar delayed fermentation of lactose have been encountered in certain foodstuffs (Bronfenbrenner and Davis, 1918; Koser, 1920).

Where lactose fermenters were found to be present in a soil culture after enrichment, only one strain from each sample was kept for further study unless distinct differences in the Endo colony, growth, etc., were apparent. In these cases one representative of each type was isolated and retained. Seventy soil specimens were examined and from them 72 cultures of the colon-aerogenes group were obtained. Seventeen soil samples gave negative results, 36 yielded one culture each, 15 two cultures each and from each of 2 samples three strains were obtained. In those cases where two or more different cultures were found in the same sample, subsequent investigation showed that the cultures frequently belonged to different sections of the group as brought out by the differential tests.

Descriptions of the media and procedures employed in the present investigation are given below.

Citrate medium. For the test of citrate utilization a chemically definite medium containing certain inorganic salts, an inorganic source of nitrogen, and a citrate as the only source of carbon was employed. Several different combinations may be used. Two of the most convenient are given here. [1] 5 grams NaCl, 0.2 gram $MgSO_4$, 1 gram (NH_4) H_2PO_4, 1 gram K_2HPO_4, and 2 grams sodium citrate (2.77 grams sodium citrate. $5\frac{1}{2}$ H_2O) in 1000 cc. of distilled water. [2] 1.5 grams Na (NH_4) HPO_4 + 4 H_2O (microcosmic salt), 1 gram KH_2PO_4, 0.2 gram $MgSO_4$, and 2 grams sodium citrate in 1000 cc. of distilled water. The hydrogen-ion concentration of both of these media is about 6.7 to 6.9. Potassium citrate may be substituted for the sodium citrate. Ammonium citrate constitutes a convenient combination of a source of nitrogen with the citrate radical, but has the disadvantage of being a very unstable salt.

The above media were tubed in 5 to 8 cc. quantities, sterilized in the autoclave and inoculated directly from plain agar slants of the colon-aerogenes cultures. The temperature of incubation was 30°C. Observations of growth, as shown by visible turbidity, were made at intervals of one, two, three, four, and seven days and where negative results were obtained after two and three weeks.

Uric acid medium. The composition of the uric acid medium was the same as that previously described (Koser, 1918). Since development in this medium depends upon the presence of available nitrogen some care must be exercised to exclude free ammonia in so far as possible. Also the medium should be inoculated lightly from young agar cultures in order to avoid carrying over any superfluous quantity of dead cells, enzymes, etc. In one set of experiments 0.2 per cent glucose was substituted for the 3 per cent glycerol as the source of carbon, since it was though that perhaps glycerol might not be readily utilized by some of the colon group cultures. However, similar results were secured in both cases. The temperature of incubation and the method of observing development were the same as those described for the citrate medium.

Methyl red and Voges-Proskauer tests. Cultures for these tests were grown for four days at 30°C. in the medium recommended by Clark and Lubs (1915)—0.5 per cent Witte pepton, 0.5 per cent dipotassium phosphate and 0.5 per cent glucose. Care should be taken to secure a dipotassium phosphate of known purity. A dilute solution of K_2HPO_4 should give a deep red to phenol red or a light pink to phenolphthalein. Details of the technic of these tests given in the American Public Health Association Standard Methods (1920) were followed with one exception. The Voges-Proskauer tests, after addition of the 10 per cent potassium hydroxide solution, were incubated at 37°C. for five or six hours and after recording the results at this time they were held overnight at room temperature and again examined to detect any additional positive tests.

Every culture was run through each of the differential tests—methyl red, Voges-Proskauer, citrate and uric acid media—several

times to determine whether there would be any variation in these tests when repeated at different intervals. As will be shown later, a few cultures showed variation in the results of the methyl red and Voges-Proskauer tests. These cultures were tested repeatedly—ten to fifteen times or occasionally more. In summarizing the work in tables 1 and 2, all cultures are recorded as definitely positive or negative in the four differential tests only if the results were consistent upon several different occasions. The production of visible growth in the synthetic media is given at the expiration of three days, since with one or two exceptions all cultures capable of development in these media produced a definite turbidity within twenty-four or forty-eight hours, and therefore the three-day interval appears adequate for routine purposes.

Table 1 presents the results obtained with the colon group cultures from fecal specimens. It is evident that growth in the citrate and uric acid media correlates almost perfectly with the methyl red and Voges-Proskauer tests. Of 109 methyl red positive, Voges-Proskauer negative cultures from human and animal feces, 107 failed to develop in the citrate medium while 108 gave negative results in the uric acid medium. Of the two methyl red positive cultures which utilized citrate, one could not be considered as a typical *Bact. coli* since its cultural and fermentative properties resembled *Bact. aerogenes*. Also, this strain produced a luxuriant raised pink colony on Endo plates and developed readily in the uric acid medium. One additional culture exhibited a delayed utilization of citrate, producing visible turbidity regularly after five to seven days. This was the only instance in which a result of this kind was encountered. Nine cultures of the aerogenes type were all capable of rapid and luxuriant growth in both the uric acid and citrate media.

The soil cultures are shown in table 2. Here it will be seen that all the aerogenes-cloacae types developed readily in the citrate medium and that this property was correlated with alkalinity to methyl red, a positive Voges-Proskauer reaction and in most cases with growth in the uric acid medium. A

TABLE 1

Cultures from fecal specimens

SOURCE OF CULTURES	NUMBER OF CULTURES	PEPTONE, GLUCOSE, K_2HPO_4 (4 DAYS AT 30°C.)		GROWTH AFTER 3 DAYS AT 30°C.		REMARKS	REFERENCES TO TABLE 3
		Methyl red	Voges-Proskauer	In citrate medium	In uric acid medium		
Typical fecal coli							
Human	30	+	0	0	0		
Sheep	8	+	0	0	0		
Fox	1	+	0	0	0		
Dog	13	+	0	0	0		
	1	+	0	0*	0		A
Cat	8	+	0	0	0		
Rabbit	26	+	0	0	0		
Rat	13	+	0	0	0		
Guinea-pig	7	+	0	0	0		
	1	+	0	+	0	Typical *Bact. coli.*	
	108						
Atypical							
Rabbit	1	+	0	+	+	Endo colonies and cultural properties similar to aerogenes type	B

Fecal aerogenes

Rabbit	3	0	+	+	+		
Guinea-pig	3	0	+	+	+		
	2	0	Weak + or 0	+	+	Cultural properties resembling *Bact. aerogenes*	
Human	1	0	Weak + or 0	+	+		
	9						
Total	118	+ = 109 0 = 9	0 = 109 + = 6 Var. + = 3	0 = 107 + = 11	0 = 108 + = 10		

Of 118 fecal cultures:

107 (90.7 per cent) failed to develop in the citrate medium

108 (91.5 per cent) failed to develop in the uric acid medium

109 (92.4 per cent) were methyl red positive and Voges-Proskauer negative

* Delayed in growth in citrate medium, becoming + regularly after five to seven days.

TABLE 2

Soil cultures

NUMBER OF CULTURES	PEPTON GLUCOSE K_2HPO_4 (4 DAYS AT 30°C.		GROWTH AFTER 3 DAYS AT 30°C. IN		REMARKS	REFERENCE TO TABLE 3
	Methyl red	Voges-Proskauer	Citrate medium	Uric acid medium		
36	0	+	+	0 or ? = 7 s.l. + = 3 + = 26	Typical aerogenes-cloacae types	
1	0	Weak + or 0	+	Sl. +	Cultural and fermentative properties resembling aerogenes-cloacae types	
2	Neutral tints	+	+	+		
8	Var.*	Var.*	+	+	Cultural and fermentative properties resembling aerogenes-cloacae types	C1 to C8
23	+	0	+	0 or ? = 20 Sl. + = 2 + = 1	(See table 3 for details)	D1 to D23
2	+	0	0	0	Resembling typical *Bact. coli.*	E1, E2
72	0 = 37 ? or var. = 10 + = 25	+ = 38 Var. = 9 0 = 25	+ = 70 0 = 2	+ or s.l. + = 43 0 or ? = 29		

*Of 72 soil cultures:

70 (97.2 per cent) utilized citrate and in this respect were distinct from the fecal Bact. coli type.

43 (59.7 per cent) developed in the uric acid medium.

38 (52.8 per cent) were Voges-Proskauer positive and 37 (51.4 per cent) were alkaline to methyl red, consistently and upon repeated tests.

*8 (11.1 per cent) exhibited a change from the methyl red positive, Voges-Proskauer negative type when first isolated to a methyl red negative, Voges-Proskauer positive reaction upon later cultivation (see text for details).

25 (34.7 per cent) were consistently methyl red positive and Voges-Proskauer negative.

number of cultures which gave irregular or variable results with the methyl red and Voges-Proskauer tests were all capable of utilizing both citrate and uric acid. The general cultural and fermentative properties of these organisms resembled those of the aeorgenes-cloacae section of the group. In addition to these types there were 25 soil cultures which were consistently methyl red positive and Voges-Proskauer negative. Twenty-three of these failed to show the usual correlation between the differential tests, for they were methyl red positive and also developed readily in the citrate medium. The ability to utilize citrate exhibited by the methyl red positive organisms from soil is noteworthy and presents a decided contrast to the negative results given by the methyl red positive strains of fecal origin shown in table 1.

The large number of methyl red positive colon group organisms from soils which were considered free from human pollution was quite surprising. They constituted 34.7 per cent of the total number of soil cultures, a higher proportion than usually reported. Johnson and Levine (1917) found that 19.7 per cent of the lactose fermenters from field and garden soils belonged to the coli section. Burton and Rettger (1917) isolated 193 colon-aerogenes cultures from soil, leaves, twigs, etc., and found only 36, or 18.7 per cent, which resembled *Bact. coli*. Chen and Rettger (1920) reported that 20 of 467 soil strains, or 4.3 per cent, belonged to the methyl red positive type. In a study of colon-like bacteria found on grains, Rogers, Clark, and Evans (1915) encountered a few cultures which gave a CO_2:H_2 ratio similar to that of the fecal *Bact. coli* but which differed from the characteristic fecal cultures in that they formed a light yellow pigment.

To determine if possible to which section of the group the several variable and irregular cultures might be related, their cultural and fermentative properties were further studied. The detailed results secured with the most perplexing organisms are given in table 3. It should be noted here that all irregular cultures were replated and reisolated several times in an effort to eliminate contaminants as the cause of the irregularity. No contaminating organisms could be found and the cultural charac-

TABLE 3

Irregular and variable cultures

REFERENCE FROM TABLES 1 AND 2	FERMENTATION OF						GELATIN LIQUEFACTION	INDOL	ENDO COLONIES
	Lactose	Salicin	Sucrose	Raffinose	Dextrin	Adonitol			
Fecal origin									
A	+	+	0	0	0	0	0	+	Coli-like
B	+	+	+	+	+	+	0	0	Large mucoid, pink
Soil origin									
C1	+	+	+	+	0	+	0	0	Large pink
C2	+	+	+	+	0	+	0	0	Large mucoid, pink
C3	+	+	+	+	0	+	0	0	Large pink
C4	+	+	+	+	0	+	+	0	Large mucoid, pink
C5	+	+	+	+	0	+	0	0	Large pink
C6	+	+	+	+	0	+	0	0	Large pink
C7	+	+	+	+	0	+	0	0	Large pink
C8	+	+	+	+	0	+	0	0	Large pink
D1	+S	+	+	+	0	0	+	0	
D2	+	+	+	+	0	0	0	+	With the exception of
D3	+	+	+	+	0	0	0	+	the slow lactose fer-
D4	+	+	+	+	0	0	0	+	menters, colonies
D5	+	+	+	+	0	0	0	+	were usually quite
D6	+	+	+	+	+	0	0	+	similar to those of
D7	+	0	0	0	0	0	0	0	typical fecal *Bact.*
D8	+S	0	0	0	0	0	0	0	coli. Flat, deep red
D9	+	+	0	0	0	0	0	0	and occasionally show-
D10	+	0	0	0	0	0	0	0	ing metallic lustre
D11	+	0	0	+	0	0	+	0	
D12	+	+	+	+	0	0	+	+	
D13	+S	+	0	0	0	0	+	0	
D14	+	0	0	0	0	0	+	0	
D15	+	0	0	0	0	0	+	0	
D16	+	0	0	0	0	0	0	0	
D17	+	0	+	+	0	0	0	0	
D18	+S	+	0	0	0	0	0	0	
D19	+S	+	0	0	0	0	0	0	
D20	+S	+	0	0	0	0	0	0	
D21	+	0	0	0	0	0	0	0	
D22	+	+	+	+	0	0	0	+	
D23	+	0	0	0	0	0	0	0	
E1	+	+	0	0	0	0	0	+	Coli-like
E2	+	0	0	0	0	0	0	+	Coli-like

Delayed lactose fermentation is designated as +S.

teristics exhibited by the newly isolated cultures were the same as those of the old cultures.

Among the irregular cultures several appear as especially noteworthy. Thus, one of the strains obtained from rabbit feces (B—tables 1 and 3) shows the cultural and fermentative properties of *Bact. aerogenes*, although the results of the methyl red and Voges-Proskauer tests were regularly those of *Bact. coli*. This culture produced a luxuriant growth in both citrate and uric acid media. Eight cultures (C1 to C8, tables 2 and 3) from soil exhibited an interesting reversion in the methyl red and Voges-Proskauer tests. When tested immediately after isolation these cultures were all methyl red positive and Voges-Proskauer negative upon incubation at 30°C. for four days. After cultivation upon agar slants in the laboratory for a month or more they became methyl red negative and Voges-Proskauer positive. This change was gradual and not abrupt. Repeated tests during this time showed a transition period during which the culture would exhibit a neutral tint to methyl red with the Voges-Proskauer test negative or questionable after four days at 30°C., whereas when tested after seven days at 30°C. the reaction would be of a more definite methyl red negative, Voges-Proskauer positive type. Finally, after further cultivation, these cultures always gave a definite methyl red negative, Voges-Proskauer positive test after four days at 30°C. and then appeared to be fixed since further variation was never observed. During the transition period, when it became evident that the methyl red and Voges-Proskauer tests were not checking with previous tests, these cultures were replated upon Endo medium and well-isolated colonies were again transferred to agar slants. All of the colonies appearing upon the Endo plates presented the same appearance, with no evidence of contaminations. The old cultures were retained along with the newly isolated ones and thereafter the two sets from each of the eight strains were tested side by side in the glucose, pepton, dipotassium phosphate medium, in citrate medium and in uric acid medium. No difference was apparent in the behavior toward citrate and uric acid. Results with the methyl red and Voges-Proskauer tests

showed no difference between the newly isolated and the older cultures since both exhibited the transition to the methyl red negative, Voges-Proskauer positive type of reaction.

While it is realized that contaminations cannot be positively excluded in these cases, nevertheless it is believed that the change in the tests as observed cannot be accounted for in this manner. It seems probable that as a result of laboratory cultivation the secondary or alkaline fermentation was "speeded up" until finally the reversion of reaction to give an alkaline test to methyl red took place within the customary time of four days. The production of acetyl methyl carbinol, as shown by a positive Voges-Proskauer test, was never apparent until this reversion had taken place. It will be seen from tables 2 and 3, C1 to C8, that the eight cultures which exhibited this peculiarity evidently belonged to the aerogenes-cloacae section of the group as judged by carbohydrate fermentations, Endo colonies, and by the utilization of both citrate and uric acid.

Irregular and atypical cultures in regard to the deportment toward the methyl red and Voges-Proskauer tests have been encountered by practically all who have worked with the colon group of organisms. As bearing especially upon the present investigation it might be mentioned that Chen and Rettger (1920) reported that 18 cultures resembling the aerogenes type gave at first methyl red positive, Voges-Proskauer positive tests. Upon repeated plating the number of non-correlating organisms was reduced to 4, although contaminations could not be demonstrated. They further state (p. 287) "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 to 6.5." Stovall and Nichols (1918) reported variation in the methyl red and Voges-Proskauer tests when they were applied twice at six-month intervals to 200 colon group organisms obtained from water supplies in the state of Wisconsin.

As previously pointed out, the methyl red positive soil cultures form an interesting group. (See tables 2 and 3, D1 to

D23, E1, E2). The carbohydrate fermentations of several of these cultures approached those assigned to the aerogenes-cloacae section (Winslow, Kligler, and Rothberg, 1919). On the other hand, the types of colonies formed on Endo medium resembled in many instances those shown by the typical fecal *Bact. coli*. It is a curious anomaly that most of the cultures which fermented salicin, sucrose, and raffinose, and thus approached the aerogenes type, should at the same time give a positive indol test (table 3, D2, to D6, D12 and D22). Again, the cultures which did not attack this series of carbohydrates and thus resembled the coli type were usually indol negative. Several of these cultures liquefied gelatin while others exhibited the delayed fermentation of lactose. Of the 25 methyl red positive soil cultures only 2 failed to develop in the citrate medium (tables 2 and 3, E1 and E2). These two resembled the typical fecal *Bact. coli* type in every respect. One of these cultures was obtained from a Virginia soil and one from a Maryland specimen taken about twelve miles from Washington, D. C. It is not known whether they represent true soil forms or whether in spite of precautions taken in collecting the samples, they indicate chance human or, more probably, animal pollution. The various cultural and differential tests point to the latter conclusion.

To correlate the source of the cultures with the various methods used for differentiation of the colon-aerogenes group, reference should be made again to tables 1 and 2. The fecal cultures of the *Bact. coli* type form an homogenous group with very little variation in any of these tests, all of which correlate almost perfectly. The fecal aerogenes cultures all utilized citrate and uric acid readily. However, upon examining the results obtained with the soil cultures one is immediately impressed, first, by the variability of the methyl red and Voges-Proskauer tests and, secondly, by the fact that 25 of a total of 72 cultures were found to be methyl red positive and Voges-Proskauer negative. The degree of correlation of the various differential tests is given at the foot of table 2. Here we find that 70 of 72 soil cultures, or 97.2 per cent, developed in the citrate medium. Only slightly over 50 per cent were consist-

ently Voges-Proskauer positive and methyl red negative, while 25, or 34.7 per cent, of the soil cultures were consistently methyl red positive and Voges-Proskauer negative. In the uric acid medium 43, or 59.7 per cent of the soil cultures were capable of development. In the present investigation then, the ability to utilize the citrate radical, as shown by growth in a synthetic citrate medium, was found to correlate very closely with the source of the cultures. The methyl red and Voges-Proskauer tests were of less value in placing the source of the cultures, since a fairly large proportion of the soil strains were methyl red positive and Voges-Proskauer negative. In addition, some variability and irregularity was encountered in the use of these tests. Uric acid appears to be less useful than citrate for only about 60 per cent of the soil cultures developed in the uric acid medium. It should be noted that most of the methyl red positive soil cultures which utilized citrate (table 2, D) refused to grow in the uric acid medium. Also a few of the typical aerogenes-cloacae soil types failed to show development in this medium.

It has been commonly assumed that all methyl red positive, Voges-Proskauer negative cultures encountered in soil, water, etc., are of fecal origin since about 95 per cent of fecal cultures give this reaction. However, in the present investigation 34.7 per cent of the colon group cultures from soil were methyl red positive and Voges-Proskauer negative and all but two of these cultures were distinct from the typical fecal *Bact. coli* as shown by their development in the citrate medium. There is evidence, therefore, that not all of the methyl red positive colon group cultures encountered in nature are of fecal origin. There is to be found in soil, in addition to the well-recognized aerogenes-cloacae type, another section of the colon group which may be characterized as being methyl red positive and Voges-Proskauer negative and possessing the ability to utilize citrate as a source of carbon. Hitherto this section has not been separated from the fecal methyl red positive type because the citrate differentiation has not been available. A suggestion that some of the methyl red positive colon group organisms encountered in soil

and water may differ from the fecal methyl red positive type is found in several recent publications. Thus, Chen and Rettger (1920) isolated from soil 20 methyl red positive colon cultures, 10 of which developed in the uric acid medium, whereas 173 colon strains isolated from feces gave negative results in this medium. Perry and Monfort (1921), in a study of atypical colon-aerogenes forms obtained from natural waters, found a number of methyl red positive organisms which also developed in the uric acid medium.

Whether the results presented in this paper will prove to have a practical application in the estimation of the sanitary quality of water remains to be seen. The methyl red and Voges-Proskauer tests, which at one time were regarded by many as solving all the difficulties encountered in interpreting the presence of the colon group in water, seem to have lost ground in recent years. Some investigators believe that in routine analysis this differentiation has little or no practical significance, while many water laboratories at the present time do not use it at all. On the other hand, others adhere to it and claim that it is useful in certain cases. Levine (1921) states that in routine water analysis this differentiation is obviously desirable as it may assist in the detection of the probable source and nature of the contamination. In the present investigation, the test of citrate utilization to distinguish types distinct from the fecal *Bact. coli* was found to constitute an additional differential characteristic which may possess some value in clearing up the present uncertainty. It has correlated much more closely with the source of the cultures than have any of the other methods of differentiation and it seems therefore that further study of the usefulness of the citrate differentiation would be warranted, especially as applied to cultures obtained from waters of different sanitary quality.

SUMMARY

In a series of colon-aerogenes cultures, the source of the organisms was compared with the various differential tests used to separate the sections of this group. Of the 190 cultures em-

ployed, 118 were isolated from fecal specimens of both man and animals and 72 were obtained from various soil samples which were collected from locations where the chance of pollution seemed remote. The differential tests employed were, first, determination of the ability to utilize citrate as a source of carbon, secondly, the ability to utilize uric acid as a source of nitrogen and, lastly, the usual methyl red and Voges-Proskauer tests.

The results confirmed previous work in that the typical *Bact. coli* of fecal origin is unable to utilize sodium or potassium citrate when supplied as the sole source of carbon, whereas the aerogenes-cloacae types, which represented the largest section of the soil cultures, all utilized citrate readily.

Some exceptions were noted to the usual correlation between the manner of glucose metabolism, as shown by the methyl red and Voges-Proskauer tests, and the ability to utilize citrate. These discrepancies occurred chiefly among the soil cultures. Of especial interest were 23 soil strains which were consistently methyl red positive and at the same time developed in the citrate medium. These organisms seem to constitute another section of the colon-aerogenes group which may be differentiated from the fecal methyl red positive type by their utilization of citrate. Their presence in soil and apparent absence from fecal specimens constitutes evidence that not all of the methyl red positive colon group organisms encountered in nature are of fecal origin.

The citrate medium appeared to advantage in separating the various types of the colon group from the typical fecal *Bact. coli* and the test of citrate utilization correlated more closely with source than did any of the other differential tests. Thus, of the 72 soil cultures, 70, or 97.2 per cent, utilized citrate, slightly over 50 per cent were consistently alkaline to methyl red and Voges-Proskauer positive, while about 60 per cent developed in the uric acid medium.

Irregularity and variability of the methyl red and Voges-Proskauer tests were encountered in a number of cultures. Differentiation by means of the citrate medium was found to be especially useful in assigning these cultures to their proper groups as well as indicating their source.

The results obtained in the present investigation suggest that the test of citrate utilization deserves further study with respect to the distribution of the colon-aerogenes group in nature, with especial reference to possible usefulness in the estimation of the sanitary quality of water.

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