COLI-AEROGENES DIFFERENTIATION IN WATER ANALYSIS

II. THE BIOCHEMICAL DIFFERENTIAL TESTS AND THEIR INTERPRETATION

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We have shown in an earlier section of this paper that a larger percentage of routine plates may contain no typical *Bact. coli* or *Bact. aerogenes* colonies, but that these cultures are often mixed and therefore cannot be differentiated without purification. Also that a surprising portion of single isolated colonies, ordinarily considered pure and usually differentiated macroscopically, are not pure. These must be purified before they can be correctly differentiated. We have shown that neither the macroscopic examination of a single streaked plate nor the microscopic examination of a Gram stain smear gives sufficient evidence of the purity of a culture. We have relied largely on certain biochemical differential reactions used in conjunction with the aforementioned tests. The efficacy of the various methods used for purification will be determined to a large extent by the use of biochemical differential methods. We are therefore going to reverse the natural order of sequence and describe the biochemical tests used before we discuss purification.

CHARACTERISTICS FOR DIFFERENTIATION

In order to differentiate the organisms of the coli-aerogenes group it is necessary to use characteristics which are different for the two types and are also stable and not easily variable in the individual species. Bergey (1925) separates the coli section or genus *Escherichia* from the aerogenes section or genus *Aerobacter* on the basis of the acetyl-methyl-carbinol or Voges-Proskauer

reaction. Kluyver and Donker (1926) use the same reaction as a basis of separation for the group. However, Buchanan and Fulmer (1930) citing the above investigators say:

> Kluyver and Donker[1] (1926) have also shown that in a suitably buffered medium *Bacterium coli* does not transform all acetaldehyde to ethyl alcohol and acetic acid, but in part to acetyl-methyl-carbinol. In other words, a modification of the pH of a medium changes the metabolism to that characteristic of *Bacterium aerogenes.*

If the above is true, this basis of classification and separation becomes rather weak.

In his study of microbic dissociation Hadley (1927) reviews much of the work on dissociation of the coli-aerogenes group and shows that it has been repeatedly observed in members of this group. Unfortunately most of the investigators studied dissociation with respect to cultural, morphological, serological or fermentative characteristics. There is a lack of data on the other biochemical variations accompanying dissociation in the coli-aerogenes group. If a simple adjustment in the pH of the medium, as stated by Kluyver and Donker, is sufficient to enable *Bact. coli* to assume a biochemical characteristic of *Bact. aerogenes* we would certainly expect biochemical variations in dissociants of the group. Hadley has shown that there is a variation in the production of pyocyanin and in the proteolytic power of the dissociate and the mother type of *B. pyocyaneus.* On the other hand Soule (1928) reports that both the S and R forms of *B. subtilis* produce indol and reduce nitrates. The fact that Neisser (1906) and also Massini (1907) obtained a "coliform" lactose fermenting organism as a dissociant from a parent culture that could not ferment lactose is rather surprising. We have isolated a number of coliform cultures which apparently lost their power to ferment lactose after transferring to lactose and replating on an isolation medium several times. We explain this phenomenon on the basis of symbiotic gas production as shown by Sears and Putnam (1923) and Ishikawa (1927), and the final ascendency and isola-

[1] See note, page 135, for recent opinion of Kluyver and Donker.

tion of one non-lactose-fermenter of a pair in an initially mixed culture, rather than by dissociation. Dulany (1928) reported that *Bact. coli* dissociants had characteristic differences in colony form and cell morphology. Also, though a distinct difference in the virulence of the dissociants was noted, only slight differences in the cultural reactions were found. Dawson (1919) showed that by changing the character of the medium the chemical constitution of the bacteria changed. The chemical composition of the organisms showed considerable variation after they had been grown for about 200 generations on 8 different media. These changes were accompanied by variations in the serological and fermentative reactions so that in some cases the variations amounted to the production of a new strain. Hadley (1927) cites Bergstrand, Hauduroy, Fejgen, Ogata, Grumbach, and Dimtzer as having reported similar fermentative changes in dissociants. From the literature it is difficult to determine whether the more common biochemical reactions of the coli-aerogenes group are any more stable than the colonial, morphological, serological or fermentative characteristics.

Our experience has been that some of these biochemical tests are very stable, cannot be easily changed and are the most reliable characteristics for differentiation of the members of the Coli-aerogenes group. We shall therefore study the following problems in this paper:

1. The various biochemical reactions available.
2. The technique and particular value of the four tests chosen.
3. The stability of this entire group of tests on pure *Bact. coli* and *Bact. aerogenes* strains.
4. The interpretation of the various combinations of these four tests observed on routine cultures.
5. The various methods of purification tried and their value as judged by these biochemical tests for purity.

THE BIOCHEMICAL DIFFERENTIAL REACTIONS

Standard Methods (1925) recommends the methyl-red and Voges-Proskauer reactions, Koser's citrate test and the uric acid test for differentiation of the coli-aerogenes group. Many other

tests have been suggested and have value under special conditions. Some of these are the indol test, proteolytic tests, the chinic acid test (Butcher (1926)) and other organic acid tests, the cellibiose test (Jones and Wise (1926) and Koser (1926)) and the various other carbohydrate fermentation tests. The proteolytic tests and the chinic acid medium are valuable largely in the differentiation of the *Bact. cloacae* and other members of the aerogenes section. This is not so important in water laboratories where it is necessary to limit the number of tests and therefore neither of these tests were included in our routine work. While the reports on the cellibiose test have been favorable, it is rather expensive and therefore impractical for routine laboratory use. The various carbohydrate fermentation tests have long been used in systems of classification of the coli-aerogenes group. Houston (1911) obtained 26 real or apparent varieties of lactose-positive and indol-producing organisms from water when classified by such sugar fermentation tests. Levine (1921) makes the following statement in regard to these tests.

> A very serious objection to such classifications as those of MacConkey, Bergey and Deehan, and Jackson is their extreme flexibility and complexity, for as the number of fermentable substances or other characters observed increases, the number of "varieties" increases geometrically (approaching infinity) and soon produces a most unwieldy scheme.

For this reason and because the fermentable characteristics of the coli-aerogenes group seem to vary upon dissociation of these organisms we have not used any of these rare sugars. While they are interesting and no doubt important for some purposes they seem to be of little value in interpreting water quality.

Koser (1925) studied the utilization of the salts of other organic acids similar to citric acid by the coli-aerogenes group. He decided that none of them afforded the same distinction between the intestinal *Bact. coli* and other members of the group that citric acid salts did. Though both the uric acid and the citrate tests are recommended in Standard Methods these two tests are very similar and the citrate test seems to be the more reliable. We therefore selected Koser's citrate test in preference to the uric

acid test. We have also included the methyl-red test, the Voges-Proskauer reaction and the indol test for reasons that will appear later.

THE VALUE AND TECHNIQUE OF THE SELECTED BIOCHEMICAL TESTS

The methyl-red test has been and probably still is the most popular and widely used differential test in water laboratories. The test was introduced by Clarke and Lubs and is practically the same today as when originated. It depends upon the ability of the aerogenes section to attack the acids produced in their primary fermentation of glucose broth and raise the pH of a properly buffered medium and the inability of the coli section to utilize their acid end products in this fashion. It is therefore essentially a pH determination with methyl-red as an indicator in a special medium. Its success depends upon a sufficiently long incubation period to allow the differences in the complete carbohydrate metabolism of the *Bact. coli* and *Bact. aerogenes* organisms to take place.

This test is very valuable but has its limitations. Other organisms often associated with the coli-aerogenes group in water and sometimes found as contaminants of coli-aerogenes group colonies on streaked isolation plates, such as the *Cl. Welchii* group, also produce methyl-red positive tests like those of *Bact. coli*. Some soil organisms have the ability to attack organic acids in a way similar to *Bact. aerogenes* and give methyl-red negative results. Organisms such as those mentioned above, either alone or with members of the coli-aerogenes group, may produce erroneous methyl-red tests. It is also apparent that mixtures of the coli and aerogenes sections can only give one reaction. The mere presence of *Bact. coli* at the start does not assure a methyl-red positive in such cases. The result will depend upon the factors discussed in the first section of this paper. In this case, as in preliminary enrichment, the result is unpredictable. The ratio of the two strains present at the start, their lag phases, maximum growth rates and sensitivity to the products of metabolism all affect the result. In general, the shorter the incubation period, with twenty four hours as a limit, the greater is the chance for a methyl-red

positive result with coli-aerogenes mixtures. Ruchhoft, Kallas and Chinn (1931) have shown that when *Bact. coli* and *Bact. aerogenes* mixtures were planted together into glucose peptone-phosphate broth the proportion of methyl-red positives decreased as the period of incubation at 37°C. was increased from one to five days. After four days (the incubation period recommended by standard methods (1925)) only 74 per cent of the mixed *coli-aerogenes* cultures indicated *Bact. coli* by a methyl-red positive result while *Bact. coli* were recovered from 95 per cent of them. With pure *Bact. coli* strains methyl-red positive results will be obtained if the test is made at any time after the first twenty-four hour incubation period. There is however, a considerable variation in ability of different *Bact. aerogenes* strains to utilize the organic acids formed during the primary fermentation. This is shown by the different times required for various *Bact. aerogenes* strains to produce methyl-red negative tests.

The pH curves of twenty successive daily transplants into lactose broth of 2 different aerogenes strains was followed for ten days. These pH curves for the two strains were different on the initial cultures and indicated that the first one had the greater ability to utilize acid and increase the pH. These characteristic pH curves for the two strains continued throughout the experiment, the final curves for each strain being practically duplicates of the first and showing the same characteristic difference in the velocity at which the pH was increased. This experiment indicated the constancy of the fermentative reactions produced by these strains. A similar experiment on *Bact. coli* strains indicated a similar constancy and the lack of ability to utilize the organic acids. Some aerogenes strains produce methyl-red negative tests in twenty-four hours while others require up to three days or more to accomplish the same result. Methyl-red positive tests may be obtained with some pure aerogenes strains after forty-eight hours and methyl-red neutral or doubtful tests may even be obtained with such strains after seventy-two hours, and occasionally longer, at 37°C. Some investigators have considered cultures producing neutral methyl-red tests with positive Voges-Proskauer reactions as anomalous or intermediate

forms in the group. We have however found this variability in the methyl-red reaction among aerogenes strains that were regular in their inability to produce indol and ability to utilize citrate. We therefore regard such strains as normal members of an aerogenes section that has considerable variation in the organic acid utilizing velocity of the various strains.

The character of the methyl-red test and the variation in the ability of the aerogenes strains to utilize the organic acids justifies the rather long incubation period of four days at 37°C. suggested by Standard Methods. Levine (1921) believes that the incubation period necessary for an accurate differentiation with the methyl-red test is too long for a good routine test. Prescott and Winslow, (1924) considered the procedure recommended by the 1923 Standard Methods as needlessly complicated and state that:

> A single additional subculture in dextrose peptone phosphate broth for the methyl-red test would seem to be sufficient for all practical purposes; and we believe that such a test should be made where full knowledge of the sanitary quality of a water sample is desirable.

The data presented by several investigators which we will discuss later indicates that the results of the methyl-red test alone are not entirely reliable. In conjunction with other tests it is very valuable and therefore we have selected it as one of our tests.

THE VOGES-PROSKAUER TEST

The Voges-Proskauer test is also widely used in water laboratories. The reaction depends upon the ability of *Bact. aerogenes* to produce acetyl-methyl-carbinol in a peptone glucose broth with a resultant color reaction upon the addition of an alkali. Levine (1921) selected this test as the most satisfactory for routine work on account of its simplicity and the fact that the reaction could be obtained within twenty-four hours at 37°C. Chen and Rettger (1920) also have found it satisfactory and constant. It has recently been severely criticized. Several water analysts have reported it to be of no value. Linton (1924) first reported anomalous reactions with the Voges-Proskauer test and reported that acetyl-methyl-carbinol was a transient end product of glucose fer-

mentation. Paine (1927) reported the destruction of the acetyl-methyl-carbinol by certain organisms in the coli-aerogenes group. Williams and Morrow (1928) confirmed Paine's work and reported that aerobic spore forming organisms and the green fluorescent bacteria besides certain strains of *Bact. coli* and *Bact. aerogenes* could destroy acetyl-methyl-carbinol. Ruchhoft, Kallas and Chinn (1931) also reported that mixtures of *Bact. coli.* and *Bact aerogenes* could not be depended upon to exhibit the presence of *Bact. aerogenes* by the Voges-Proskauer reaction with incubation periods between one and five days at 37°C.

It must also be remembered that other organisms, particularly soil forms which may be present as contaminants of the coli-aerogenes group also give the Voges-Proskauer reaction. Such organisms have been reported by Meyer (1918), Ginter(1927) and Koser and Shinn (1927).

After a series of comparative tests we found the Digestive Ferments Company's dehydrated M.R.-V.P. medium more uniform and superior to the glucose-peptone-phosphate broth prepared in our laboratory for the Voges-Proskauer reaction. We have adopted this dehydrated medium. In making the test an equal volume of 10 per cent KOH solution is added to the cultures as recommended by Standard Methods. We have found the Standard Methods recommendation, to leave the cultures stand overnight at room temperature before making readings, unsatisfactory; and believe that some method to hasten the reaction so that the observation time can be shortened is necessary. Bedford (1929) suggested the use of a small amount (10 mgm. to 2.5 cc. of culture) of sodium peroxide before adding the KOH. This is very successful in hastening the color reaction. For routine work, however, we prefer incubating the cultures at 37° or 45°C. after the addition of KOH and making color observations after one, two and six hours. We obtain the majority of the positive reactions in less than one hour by this procedure. Recently Werkman (1930) reviewed the various methods of hastening the color production and the chemistry of the reaction. He suggests a new method of hastening the reaction which appears to be very satisfactory. Werkman's method depends upon the rapid

oxidation of acetyl-methyl-carbinol to diacetyl by the addition of ferric chloride as a catalyst. The diacetyl reacts with the peptone in a alkaline solution to produce a copper coloration. Two drops of a 2 per cent ferric hydroxide solution are added to 5 cc. of culture. Then the NaOH or KOH is added as in the usual procedure and the tube shaken. The copper color indicating a positive result usually develops within one hour. We have found that returning the tubes treated in this way to the 37°C. incubator also hastens the color formation so that the majority of the positive tests are obtained in fifteen to thirty minutes. We believe that this method has advantages and is desirable. Werkman reports that it has produced positive results when the "standard" procedure has failed to detect acetyl-methyl-carbinol. We, however, have never obtained positive reactions with Werkman's technique when such reactions were not also obtained with our regular procedure.

We have undertaken a number of experiments on some of the vagaries of the Voges-Proskauer reaction. Data have been collected on the following points:

1. The time required for the reaction and the possibilities of self destruction of the acetyl-methyl-carbinol with pure *Bacterium aerogenes* cultures isolated from water.
2. The possibility of acety-methyl-carbinol production by members of the *Bacterium coli* section.
3. The destruction of acetyl-methyl-carbinol by *Bact. coli.*

In our study of the first problem, each of 30 pure aerogenes cultures was planted into 7 tubes of the M.R.-V.P. medium and incubated at 37°C. The Voges-Proskauer tests were made on one series of cultures after various incubation periods of from one to seven days with the following results:

TOTAL NUMBER OF STRAINS	CLASS 1—NUMBER GIVING POSITIVE V.P. TEST EVERY DAY	CLASS 2—NUMBER GIVING − TEST ON FIRST DAY BUT + THEREAFTER	CLASS 3—NUMBER GIVING + TEST ON FIRST DAY BUT − THEREAFTER	CLASS 4—NUMBER GIVING + TEST FOR 2 DAYS BUT − THEREAFTER	CLASS 5—NUMBER GIVING + TEST FOR 3 DAYS BUT − THEREAFTER	TOTAL NUMBER NOT GIVING + TEST ON EVERY DAY
30	21	3	3	2	1	9

The nine cultures which did not give positive tests every day were repurified and the experiment was repeated. The three cultures in class 2 then gave positive tests every day. With the remaining cultures the results were very much as they were in the first experiment. However, three colonies were picked from strain number 23 which was in class 3, and one of these subcultures continued to give the reaction throughout the experiment. One of the other subcultures failed to give the reaction after one day as in the first experiment while the other one continued to give it for three days.

This experiment confirms the results of Linton (1924) and Georgia and Morales (1926) who reported more frequent negative V.P. results with incubation periods over three days. It confirms the work of Paine (1927) and Williams and Morrow (1928 that *Bact. aerogenes* may be self destructive of acetyl-methyl-carbinol. It also shows that it is impossible to choose a definite incubation time with the assurance that a positive V.P. reaction will be obtained for every *Bact. aerogenes* culture.

PRODUCTION OF ACETYL-METHYL-CARBINOL BY BACT. COLI

To determine the possibility of acetyl-methyl-carbinol production by *Bact. coli* 39 strains have been tested at least 20 times, during the past year, after two- and three-day incubation periods at 37°C. in the M.R.-V.P. medium with negative results. These cultures were also tested after eighteen and twenty-four hours incubation with negative results. More highly buffered media were therefore prepared by adding 2, 4, 8 and 16 grams per liter of K_2HPO_4 to the standard medium.

Five *Bact. coli* strains were planted into these with two *Bact. aerogenes* controls. The pH of all cultures was followed and the Voges-Proskauer tests were made after one, two, three, five and ten days at 37°C. The *Bact. aerogenes* produced positive reactions in all of these media except the most highly buffered one, but not a single instance of acetyl-methyl-carbinol production was obtained with any of the *Bact. coli*, either with our regular technique or Werkman's. We believe that the possibility of acetyl-methyl-carbinol production by the *Bact. coli* section as

intimated by Kluyver and Donker (1926) is very remote.[2] However, the reversion of certain soil forms from M.R. + V.P. − upon isolation to M.R. − and V.P. + after several weeks cultivation as described by Koser (1924) may be interpreted as the production of acetyl-methyl-carbinol by *Bact. coli*. Speaking about such reversion Koser says:

> While it is realized that contamination 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 more 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.

Eight of Koser's cultures which exhibited such reversions in their methyl-red and Voges-Proskauer reactions belonged to the aerogenes section when judged by their ability to utilize citrate and uric acid, their carbohydrate fermentations and Endo colonies and their inability to produce indol. We conclude therefore that with the standard Clark's glucose broth or the Digestive Ferments Company's M.R.-V.P. medium the possibility of acetyl-methyl-carbinol production by *Bact. coli* may be safely ignored in water analysis.

DESTRUCTION OF ACETYL-METHYL-CARBINOL BY BACT. COLI

Whether *Bact. coli* will affect the finding of acetyl-methyl-carbinol in mixed culture with *Bact. aerogenes* may depend upon several factors. It may depend upon the ability of the *Bact. coli* to destroy acetyl-methyl-carbinol after it has been produced, or it may depend upon the growth rates of the two organisms in Clark's medium and the ascendency of the *Bact. coli* and its products of metabolism in sufficient quantity to prevent the proper secondary metabolism of the *Bact. aerogenes* with its accompanying acetyl-methyl-carbinol production.

[2] In a recent letter from Dr. Kluyver he said that the statement regarding the production of acetyl-methyl-carbinol by *Bact. coli* was based on one apparently successful result in 1926. Since that time they have repeated their experiments without success. He has not had the opportunity to withdraw the original statement since and asked us to explain this situation for him.

To determine the ability of *Bact. coli* to destroy acetyl-methyl-carbinol an acetyl-methyl-carbinol medium was produced by growing a *Bact. aerogenes* strain in glucose broth for forty-eight hours at 37°C. This culture which contained acetyl-methyl-carbinol was divided into three portions of about 1 liter each.[3] The first portion was tubed and sterlized and contained the killed cells of *Bact. aerogenes*. The *Bact. aerogenes* cells were filtered out of the other two portions and one half of 1 per cent of dehydrated M.R.-V.P. medium was added to the third portion. These three different acetyl-methyl-carbinol media were tubed and sterilized. Thirty-eight *Bact. coli* strains were planted into tubes of each medium. The cultures and control tubes were incubated at 37°C. In the first two media, where additional M.R.-V.P. medium had not been added, all cultures remained methyl-red negative during the entire experiment and there was destruction of acetyl-methyl-carbinol in only 2 cases in each medium during ten days. In the filtered medium series an excellent growth of 100,000,000 or more *Bact. coli* per cubic centimeter was obtained with each of the 38 strains. This proved that the failure of more strains to destroy acetyl-methyl-carbinol in the first and second series was not due to the failure of *Bact. coli* to grow in the medium.

The results obtained with these *Bact. coli* strains in the acetyl-methyl-carbinol medium to which additional M.R.-V.P. medium has been added were as follows:

	INCUBATION TIME				
	Initial	2 days	4 days	6 days	10 days
Total number of strains	38	38	38	38	38
Number of M.R. − V.P. +	38	0	12	14	14
Number of M.R. ± V.P. +		4	4	3	0
Number of M.R. + V.P. +		34	22	20	18
Number of M.R. + V.P. ±				0	2
Number of M.R. − V.P. −				1	4

[3] Contrary to some of the published reports it was found that acetyl-methyl-carbinol was volatile. Portions of this medium were steam distilled and tests for acetyl-methyl-carbinol were obtained on the various fractions following the addition of peptone and potassium hydroxide.

Bact. aerogenes cultures that destroy acetyl methyl-carbinol destroyed it in all of these media in four to six days. One *Bact. coli* strain No. 32 also slowly destroyed acetyl-methyl-carbinol in all of these media. The experiments showed however, that few *Bact. coli* have this ability and even when they have, the destruction requires more than four days We must conclude therefore that the actual destruction of acetyl-methyl-carbinol by *Bact. coli* is rarely the cause for the vagaries of the reaction in mixed *Bact. aerogenes* cultures.

The possibility of failure to obtain the acetyl methyl-carbinol from *Bact. aerogenes* cultures due to overgrowth of *Bact. coli* is governed by factors which have been discussed in the first section of this paper. The initial ratio of the strains present and their lag phases will affect the result. The growth rates and the effect of the products of metabolism on the strains involved will also affect the result. It is therefore impossible to predict how frequently the acetyl-methyl-carbinol reaction will fail to be obtained with contaminated *Bact. aerogenes* cultures without knowing the exact conditions involved in each case. Experiments have shown, however, that this reaction may be easily interferred with and positive tests will not be obtained on nearly all occasions when *Bact. aerogenes* are present in the culture. Implantation of *Bact. aerogenes* strains into twenty-four-hour *Bact. coli* cultures in M.R.-V.P. medium showed us that under these conditions the V.P. reaction is rarely obtained.

From the foregoing study of the possibilities of interference with the Voges-Proskauer reaction, the vagaries of the test often reported in routine water work are not surprising. The purity of the cultures, the length and temperature of incubation, and the technique for hastening the reaction or making the readings all had an effect on the final result. It is therefore interesting to compare the results obtained in four laboratories with this test as shown in table 10. In view of the well known fact that the *Bact. aerogenes* have a lower death rate in streams and stored waters than the *Bact. coli* the rather low percentage of Voges-Proskauer positive reactions reported by all laboratories is rather surprising. Direct counts on our waters have indicated a much higher average

proportion of *Bact. aerogenes* than are indicated by the Voges-Proskauer tests. Our growth rate studies have not indicated that the *Bact. aerogenes* are outgrown by the *Bact. coli* during preliminary enrichment. Still, the table shows very low Voges-Proskauer results from four widely separated laboratories. Interference with the reaction by the various causes already mentioned is we believe the explanation for these low results. It will be noted that the proportion of positive Voges-Proskauer tests was almost doubled in our laboratory from 1928 and 1929 to 1930. We do not ascribe this to a real difference in the proportion of *Bact. aerogenes* cultures isolated. We believe it is the result of an improved medium, a shortening of the incubation period and an improved technique.

We therefore conclude that, due to the high incidence of contaminated cultures and irregularities of the *Bact. aerogenes* strains, the Voges-Proskauer test is not of much value as carried out at present in water laboratories. If it is to be made at all on routine cultures it should be repeated at least twice after various incubation periods such as one and three days. Its correlation with the methyl-red-test for reasons shown is one of its weak points in routine work. For this reason the results of the methyl-red and Voges-Proskauer reactions are often misleading. These two tests should not be used alone in water analysis. When they are used with the other two tests to be described, however, they are of some value.

KOSER'S SODIUM CITRATE TEST

Koser (1923) first studied the ability of organisms of the coli-aerogenes group to utilize salts of citric acid as the only source of carbon. He made a very careful study of strains obtained from animal feces and soils and discovered that the methyl-red positive, Voges-Proskauer negative section of the coli-aerogenes group could be separated into two divisions, dependent upon their ability to utilize sodium or potassium citrate. Fecal methyl-red positive, Voges-Proskauer negative strains were unable to utilize citrates. Soil methyl-red positive, Voges-Proskauer negative cultures were able to utilize citrates.

The following table illustrates the significance of Koser's findings:

SOURCE OF CULTURE	REACTIONS			E.M.B. COLONY TYPE
	M.R.	V.P.	Citrate	
Soil	+	−	+	Coli or aerogenes type (see fig. 2)
	−	+	+	Aerogenes type
Human and animal feces	+	−	−	Coli type

In other words there were both M.R. positive, V.P. negative organisms and M.R. negative, V.P. positive organisms in soil which were alike in their ability to utilize citrate. The ability of the organisms to utilize salts of citric acid serves therefore as an index of their probable source superior to methyl-red and Voges-Proskauer tests.

The value of this test was soon recognized and it was included among the recommended differential tests in the Standard Methods (1925). Since the test was comparatively new no statement regarding the interpretation of the test was included in the 1925 edition of Standard Methods. This led to confusion in interpretation of the results obtained with it in water laboratories. Bardsley (1926) in England used this test in conjunction with the M.R., V.P. and indol tests and, apparently, because it failed to correlate as expected with these tests, dropped the citrate test as the least valuable. Antithetical opinions are found in the American literature. One investigator relied solely on the "Citrate" test in studying the incidence of fecal *Bact. coli* strains. This, of course, is poor practice because growth in citrate solution proves the presence of *Bact. aerogenes* or other citrate utilizers but it does not prove that *Bact. coli* are not also present.

Simmons (1926) and Lewis and Pittman (1928) modified the original Koser's medium, principally by the addition of agar so that the test can be made on streaked plates. Tonney and Noble (1930) confirmed 1919 cultures in both Lewis and Pittman's ferric-ammonium citrate agar and in Koser's sodium citrate solution. Of these 1919 cultures, 630 were reported as citrate utilizers in Koser's medium while only 352 were reported as

citrate utilizers on solid ferric-ammonium citrate agar. This shows that the liquid medium is superior to the solid medium in the detection of citrate utilization. Our studies of these media have confirmed the above results. We, therefore, prefer, and use, the liquid medium as described in standard methods for routine tests.

The Koser's citrate medium has characteristic advantages and disadvantages that do not appear to have been sufficiently emphasized. We will therefore discuss briefly the following points which we have learned in the course of some 9000 citrate tests made during the past year.

1. Growth of the coli-aerogenes group in the solution.
2. Effects of products of metabolism and viability of cultures.
3. Deoxygenation of the medium as a criterion of difference between the two sections of the group.
4. Changes in the pH of the medium by the coli-aerogenes group.
5. Possibility of purification by use of the medium.

GROWTH OF THE COLI-AEROGENES GROUP IN KOSER'S CITRATE SOLUTION

Our studies have indicated that all members of the coli-aerogenes group multiply in the Koser's citrate medium. The *Bact. coli* section, as reported by Koser (1923), is unable to produce a visible growth or turbidity in the medium within five to seven days at 37°C. This has been observed on more than 2000 cultures during the last two years. Thirty-eight *Bact. coli* strains isolated from water were transfered to Koser's medium and observations were made daily for five days at 37°C. and thereafter weekly for nine weeks at room temperature. At the end of one week no cultures had visible growth. After two weeks 3 strains still had no evidence of growth. The remainder of the cultures were also clear but contained slight sedimentary deposits of bacterial cells. If these tubes were shaken the sediment was dispersed and a slight turbidity appeared. Such slight turbidity, noticed only after shaking and after a week or more of incubation, is typical of the growth of *Bact. coli* and some other organisms in citrate

solution. Such growth may be confused by some with *Bact. aerogenes* but we have always found that the *Bact. aerogenes* section will produce a turbidity which is visible without shaking after shorter incubation periods. Of the 38 *Bact. coli* strains described above that were held for nine weeks, only one produced a turbidity that was visible before the tube was shaken to disperse the sediment. At the end of the experiment all of these *Bact. coli* cultures produced the same type of colonies on E.M.B. agar as when they were transferred into Koser's citrate medium. A colony from each strain that had been cultivated for sixty-three days in the citrate medium was picked and differential tests were made. All cultures had the same differential characteristics as they initially had, and were unable to produce any visible turbidity (that could be observed even after shaking the tube) after our customary incubation period of three days at 37°C. We have found that *Bact. coli* innoculums as low as one organism per cubic centimeter multiply quite rapidly at 37°C. in Koser's citrate medium until population levels of from 1 to 5 million per cubic centimeter are reached. Such population levels are often produced in twenty-four hours but are not sufficient to produce visible growth. Thereafter there seems to be very little change in the population level of pure *Bact. coli* strains. The products of bacterial metabolism in citrate medium do not seem to be of sufficient quantity, or are not sufficiently toxic, to produce any effect on the viability of *Bact. coli* strains regardless of the length of the incubation period. Therefore we have adopted the practice of holding the citrate tube culture for a week or two in routine differential studies to recover the strain if further study is necessary, regardless of whether there is visible growth. We have never lost a *Bact. coli* strain by this procedure. In fact we have recovered *Bact. coli* strains from Koser's citrate medium cultures one year old which had stood at laboratory temperatures and in which the original volume of the medium had been reduced 75 per cent or more by evaporation. These cultures were streaked on E.M.B. agar plates and usually typical colonies were obtained but in some cases rough form colony *Bact. coli* dissociants were observed.

GROWTH OF BACTERIUM AEROGENES SECTION IN KOSER'S CITRATE MEDIUM

The *Bact. aerogenes* section of the group grow more rapidly and attain higher population levels than the *Bact. coli* section. Populations of 25 to 150 million bacteria per cubic centimeter are usually obtained in twenty-four hours at 37°C. Thereafter, there is little change in the population in the ordinary cotton plugged culture tubes. Such populations are easily visible as a turbidity throughout the tube. A record was kept of the time required for 289 *Bact. aerogenes* strains to produce a turbidity throughout the citrate medium. Of these, 270 or 93.5 per cent gave visible growth in twenty-four hours at 37°C., 11 or 4 per cent after forty-eight hours and the remaining 8 or 2.5 per cent in seventy-two hours.

Tests by the dilution method, using lactose broth, tryptophane broth and citrate medium, showed that the latter medium was practically as sensitive in producing growth from very small innoculums of *Bact. aerogenes* as the two former. This confirms Koser's report (1923) on the reproduction sensitivity of the medium for *Bact. aerogenes.* We noted, however, that with such small innoculums the period required to produce a distinctly visible growth was sometimes lengthened to between twenty-four and forty-eight hours where larger innoculums produced visible growth in twenty-four hours. In routine differential work, however, the innoculum is usually large when the strain happens to be pure *Bact. aerogenes.* When the culture or colony picked contains principally *Bact. coli,* with *Bact. aerogenes* as contaminants, the innoculum may contain only a few aerogenes and it is these cases which sometimes only produce visible growth after forty-eight hours in the citrate medium.

Our routine cultures were formerly incubated for five days but the incidence of additional positive growth cultures between the third and fifth days was so low that we believe three days is long enough for routine work. We have noted that *Bact. aerogenes* can be transferred daily with a needle from tube to tube of Koser's citrate up to 15 transfers (length of our experiment) and positive growth will always be obtained in twenty-four, and at most forty-eight hours. The same treatment with *Bact. coli* cultures

never produces visible growth within three days though viable organisms are found after one day incubation of the fifteenth transfer. We have obtained routine cultures, however, which upon three days incubation produced very slight turbidities by dispersion of sedimentary cells when shaken. When such cultures were transferred to other citrate tubes no evidences of turbidity occurred in the same incubation time. Recovery of the organism by streaking on E.M.B. agar and differentiation of fished colonies showed apparently pure *Bact. coli.* It is probable that the original growth was due to another organism that was lost during this process. It may also be well to point out here that many of the positive growth cultures obtained in citrate medium in three days may be due to soil forms that are capable of utilizing citrate and are not members of the group but are often found with the coli-aerogenes forms.

EFFECTS OF PRODUCTS OF METABOLISM AND VIABILITY OF CULTURES IN KOSER'S CITRATE SOLUTION

One important characteristic of this medium is the apparent lack of effect of the products of metabolism of one strain of aerobic organisms upon any other aerobic organisms that are growing in it at the same time. This seems to be true for mixtures of *Bact. coli* and *Bact. aerogenes* strains, for *Bact. coli* and the extraneous soil forms utilizing citrate and for other groups. Unfortunately we have not studied the fate of Cl. Welchii group organisms when they are introduced into the medium mixed with *Bact. coli.* We know, however, that the *Bact. coli* reduce the dissolved oxygen content of this medium very little in cotton stoppered tubes.

When *Bact. coli* and *Bact. aerogenes* are grown together in Koser's citrate medium both strains can usually be recovered even after very long incubation periods. Sixteen different mixtures of *Bact. coli* and *Bact. aerogenes* strains were introduced into Koser's citrate medium and both strains were recovered from all mixtures at weekly intervals for five weeks. Eight of these mixtures were examined again after standing eleven months at room temperature and both strains were found in seven of them while in the eighth only *Bact. aerogenes* was recovered. These

and many other experiments all indicated the remarkable viability of both sections of the coli-aerogenes group in Koser's citrate solution. All the experiments so far described show that *Bact. aerogenes* multiplies easily rapidly producing visible growths while *Bact. coli* grows more slowly and never reaches visible population levels in the citrate medium. Unfavorable conditions are not produced in the medium by either section of the group which prevent the multiplication of the section initially in the minority to its normal population level. This makes the detection of *Bact. aerogenes* present as contaminants of *Bact. coli* easy and certain in citrate solution while it is difficult and uncertain to detect such contaminants by means of the V.P. test in Clark's broth.

DEOXYGENATION AND pH CHANGES IN KOSER'S CITRATE SOLUTION

The production of visible growth is not the only criterion distinguishing the *Bact. coli* and *Bact. aerogenes* sections of the group when grown in citrate medium. Two other changes take place in this medium which may be used to differentiate these sections. These changes are the rate of abstraction of dissolved oxygen from the medium and the changes in hydrogen ion concentration in the medium.

When *Bact. aerogenes* is grown in sodium citrate medium in completely filled glass stoppered bottles complete depletion of the dissolved oxygen in the medium usually takes place in twenty-four hours at 37°C., while with the *Bact. coli* section there is but slight reduction of the dissolved oxygen during the same time. Details of these experiments cannot be given in this paper. While the method is rather impractical for routine purposes it seems to be capable of showing slight differences in the citrate utilization ability of the coli-aerogenes group strains.

The pH of Koser's citrate medium cultures may be followed more easily than the dissolved oxygen content. Using the method of Hurwitz and Kraus (1929) we have made pH determinations on a number of coli-aerogenes group strains with the results shown in table 2. This checks Koser's (1923) results and shows that the pH resulting from a three or four days' growth of these

TABLE 1

Comparison of results of Voges-Proskauer tests on cultures from water by several observers employing various techniques

	OBSERVER				
	Bahlman and Sohn (1924)	Bardsley (1926)	Lewis and Pittman (1928)	(S.D.C.) Kallas, Weed, Berberich and Clark (1928) (1929)	(S.D.C.) Kallas, Chinn, Coulter and Luthardt (1930)
Type of water	Ohio River: raw, settled, filtered and chlorinated	Potable and polluted	Polluted and high sanitary quality waters	Lake Michigan raw and chlorinated	Lake Michigan raw and chlorinated
Medium	Clark's prepared in laboratory	Clark's prepared in laboratory	Clark's prepared in laboratory	Clark's prepared in laboratory	Clark's Difco M.R.V.P.
Incubation temperature	30°C.	30°C.	30°C.	37°C.	37°C.
Incubation period	5 days	3–5 days	5 days	4 days	3 days
Treatment previous to reading	37°C. for 8 hours		Chen and Rettger, 30°C. for 3 hours	Standard Methods, 1925	37°C. for 1 to 6 hours
Total cultures	1,223	1,441	320	1,365	1,022
Number of V.P. positive	341	180	53	203	310
Per cent V.P. positive	27.8	12.5	16.6	14.8	30.6

TABLE 2

pH obtained in Koser's citrate medium using 38 Bact. coli and 26 Bact. aerogenes strains

	INCUBATION PERIOD				
	1 day	2 days	3 days	4 days	6 days
Average *Bact. coli*	6.82	6.75	6.74	6.60	6.80
Average *Bact. aerogenes*	7.43	7.60	7.70	7.80	8.23
Maximum *Bact. coli*	6.90	6.90	6.80	6.60	6.80
Maximum *Bact. aerogenes*	7.80	7.80	7.90	8.20	8.40
Minimum *Bact. coli*	6.80	6.60	6.70	6.60	6.80
Minimum *Bact. aerogenes*	7.00	7.40	7.50	7.60	8.10
Maximum *Bact. coli*	6.90	6.90	6.80	6.60	6.80
Minimum *Bact. aerogenes*	7.00	7.40	7.50	7.60	8.10

TABLE 3

Incidence of citrate utilizing organisms from various sources (determined in Koser's citrate medium)

	SOURCE							
	Human and animal feces		Polluted water		High sanitary quality water		Soils or cereals	
	Cultures tested	Citrate +	Cultures tested	Citrate +	Cultures tested	Citrate +	Cultures tested	Citrate +
Koser (1923) (1924)	118	11	107	38	90	75	72	70
Pawan (1925)	432	16	210	18	240	195	214	193
Bardsley (1926)			979	140				
Raghavachari (1926)			1,074	331	500*	248*		
Lewis and Pittman (1928)			83	22	237	156		
Tonney and Noble (1930)	1,256	162	377	249			286	229
Houston (1928)	92	0						
Hicks (1927)	150	16						
Brown and Skinner (1930)			153	50				
Sanitary District of Chicago (1930)	486	28	2,872	1,575				
Total of above	2,534	233	5,855	2,423	567	426	572	492
Per cent citrate +		9.2		41.5		75.0		86.0

* Filtered water samples. These are not included in the high sanitary quality water total.

organisms in citrate medium is also sufficient to differentiate them. The pH changes induced by the M.R. positive, V.P. negative, citrate positive soil forms described by Koser were also determined. Six such strains were used and the results obtained were similar to those for the *Bact. aerogenes* group given in table 2.

Before concluding our study of the citrate medium we wish to point out its possible use in purification of mixed coli-aerogenes group cultures. Due to the lower population levels maintained in citrate medium streaked plates from the medium usually produce well distributed isolated colonies. Both *Bact. coli* and *Bact. aerogenes* will be produced on such plates from mixed cultures and complete separations can be more easily made than from plates streaked with cultures from media maintaining higher population levels.

Some results that have been obtained with the Koser's citrate on coli-aerogenes group isolations are summarized in table 3. The scarcity of citrate utilizing organisms in human and animal feces is strikingly shown. The greatest variation in percentages of citrate utilizers from polluted waters is illustrated in the results of Bardsley and Tonney and Noble. Bardsley found scarcely any more citrate utilizers in polluted water than are usually reported in feces. Tonney and Noble found the percentage of citrate utilizers almost as great in surface water and sewage as is usually reported in waters of high sanitary quality. Our results on polluted water indicate that at times there might be considerable variation in the percentage of citrate positive organisms isolated. The fact that there is no great difference in the percentage of citrate positive organisms isolated from raw sewage and from surface waters not highly polluted decreases the value of the test as a criterion of pollution. We found that the incidence of citrate utilizing organisms is usually higher in cultures which are reported as mixed, with or without spores, by Gram stain examination. The fact that the citrate test is a negative test for pollution and does not show that organisms of fecal origin are not also present decreases the value of the test alone. It is valuable, however, as an aid in the classification of strains and also as an index of probable mixed strains. It is our conclusion, therefore, that this

test should not be used alone but should be used in conjunction with other tests to be of the greatest value.

THE INDOL TEST

We did not adopt the indol test until a study of more than 1,000 cultures with the three tests already described had convinced us that more information was necessary for an intelligent interpretation of a great many of the results. This test was formerly considered one of the best for the identification of fecal organisms and was required for the identification of the group in the (1905) edition of the Standard Method. Castellani and Chambers (1918) used the indol reaction as a basis of classification of the Genus Escherichia and reported 17 indol positive species and one negative species. Levine (1921) found that the indol reaction correlated well with fermentation in salicin but not with dulcitol and glycerol. He used the M.R., V.P., and uric acid reactions and fermentation in sucrose, salicin and glycerol as a basis of classification of the coli-aerogenes group and did not use the indol reaction. Bergey (1925) reported that 16 of 22 species of the genus *Escherichia* and 2 out of 5 species of the genus *Aerobacter* produce indol. Jordan (1928) stated that *Bact. aerogenes* regardless of origin may produce indol. Blumenberg (1928) reported that indol production is not a constant characteristic of *Bact. coli* and that when used as a test for fecal contamination pseudo negatives may be obtained. He also reported that *Bact. cloacae* may produce indol and give pseudo positives. Nevertheless the indol test is still used and considered important by Houston (1928), Bahlman and Sohn (1924), Bardsley (1926) and Lewis and Pittman (1929) all used the indol test in conjunction with other tests with interesting results which we will discuss later.

Perry (1929) concluded that indol, methyl-red, citrate and cellibiose were the best differential tests. Hicks (1927) in Shanghai used the same tests we have used on organisms from human and animal feces and from soil. He concluded that the indol and citrate tests were of value but that the methyl-red and Voges-Proskauer tests were of no value. Raghavachari

(1926) also used these four tests and reported a very high degree of inverse correlation between the indol and citrate tests. Our experience in regard to the indol test is in accord with the results of Hicks and Raghavachari. We shall show that the indol test due to certain characteristics is very useful especially when used in conjunction with the citrate, methyl-red and Voges-Proskauer tests.

TECHNIQUE OF INDOL TEST

Kovācs (1928) described a modified technique for Ehrlich's indol test which we have found to be the best of any that we have yet tried. The reagent for the test contains 5 grams of *p*-dimethyl-amino benzaldehyde, 75 cc. of amyl alcohol and 25 cc. of concentrated hydrochloric acid. We have had the best success with Eastman's C.P. *p*-dimethyl-amino benzaldehyde and with Merck's laboratory reagent amyl alcohol. Some brands of C.P. amyl alcohol are not satisfactory, because they apparently contain furfurals which produce a dark color reaction with the *p*-dimethyl-amino-benzaldehyde. This reagent should have a yellow or light brown color when made up. This test depends upon the extraction of the indol in the culture by the amyl alcohol. The indol reacts with the *p*-dimethyl-amino benzaldehyde in the surface layer of the amyl alcohol so that the color reaction is not affected by the color of the medium.

We have found the Digestive Ferments Company's dehydrated tryptophane broth excellent for the indol test and use this medium dispensed in tubes in 4 cc. amounts for the test in our laboratory. The test is made after a twenty- to twenty-four-hour incubation period at 37°C. We have found that the addition of potassium persulphate and the 3 cc. of the *p*-dimethyl amino benzaldehyde reagent as recommended by Kovācs was not necessary. We therefore omit the potassium persulphate entirely. We simply add from 0.3 to 0.4 cc. of Kovācs amyl alcohol indol reagent to each culture, shake the tube let it stand for a minute and make the readings. We have made over 11,000 tests by this method in our laboratory during the past year with very excellent results.

SENSITIVITY OF INDOL TEST

To show the sensitivity of this indol test and the lack of interference with it by other organisms we wish to describe briefly several experiments. Thirty-eight *Bact. coli* strains (in which there were members of the following species according to Levine's classification *Bact. coli*, *Bact. acidi-lactici*, *Bact. neapolitanum* and *Bact. communior*) were used in studying the indol test. Of these, 24 gave positive indol tests after six hours incubation by the technique described above. After eighteen hours they all gave positive tests. After twenty-four hours the minimum amount of culture required to give a positive indol test was determined. Thirty-five of these strains showed a positive indol reaction with 0.1 cc. of culture after twenty-four hours while the remaining three produced weak reactions with 0.1 cc. of culture. From the 4th to the twenty-fifth day all of these strains produced positive indol reactions when 0.1 cc. of culture was added to 0.2 cc. of the indol reagent. This experiment shows the speed at which indol is formed by *Bact. coli* in this culture medium, the sensitivity of this indol test and the stability of the indol when once formed. All of these *Bact. coli* strains have been held one year on agar slants, six months in tryptophane broth, nine weeks in Koser's sodium citrate solution, they also have been repeatedly recultivated in tryptophane broth, lactose broth, glucose (Clark's) broth, brilliant green bile broth, E.M.B. agar plates and Noble's ferrocyanide citrate agar plates and they have always produced indol when reinoculated into tryptophane broth. Representatives of the above cultures were also treated in various other ways but the ability to produce indol was constant. We must therefore conclude that the ability to produce indol is a very stable characteristic of *Bact. coli* strains that cannot be easily changed by laboratory treatment.

NON-INTERFERENCE OF OTHER ORGANISMS WITH THE INDOL TEST

Another valuable characteristic of the indol test is the fact that it is not interfered with by other organisms. We have shown that in the alkaline tryptophane broth indol was indicated by 0.1 cc. of culture even after twenty-five days incubation with

pure *Bact. coli* cultures. Our studies indicate that with *Bact. aerogenes* and many other water organisms present with *Bact. coli* in tryptophane broth the indol produced will be present for similar lengths of time. In other words when indol is once produced it is not easily broken down by other organisms and can be easily detected. The only conceivable condition under which a negative indol test might be obtained with *Bact. coli* strains which produce indol would be when other organisms outgrow and produce conditions in the medium unfavorable for the growth of *Bact. coli.* The possibility of this condition occurring was investigated with *Bact. coli* and *Bact. aerogenes* mixtures. Seven representative *Bact. aerogenes* cultures were selected and each culture was inoculated into 15 tryptophane broth tubes which were incubated at 37°C. for twenty-four hours. Good growth as shown by turbidity was obtained in each case. Twelve culture tubes out of each series of 15 were then inoculated with 1 loop of 12 representative *Bact. coli* cultures. A control tube of each *Bact. coli* strain was also planted and then all tubes were incubated for an additional twenty-four hours. The entire set up then included 7 *Bact. aerogenes* strains of three tubes each for *Bact. aerogenes* controls, 84 different combinations of mixed *Bact. coli* and *Bact. aerogenes* cultures in which all of the *Bact. aerogenes* had a twenty-four-hour incubation period before the *Bact. coli* were introduced, and the 12 *Bact. coli* controls. The indol test was made on all these cultures after the *Bact. aerogenes* had been incubated for forty-eight hours and the *Bact. coli* for twenty-four hours with the results shown below:

	ORGANISMS		
	Bact. coli	*Bact. aerogenes* and *Bact. coli* mixtures	*Bact. aerogenes*
Number of strains	12	84 different combinations	7 strains, 3 cultures of each
Incubation time	24 hours	48 hours for *Bact. aerogenes*, *24 hours Bact. coli*	48 hours
Indol results	All +	79+, 3±, 2−	All −

All of the *Bact. coli* controls were indol positive and all *Bact. aerogenes* controls indol negative. Seventy-nine of the 84 mixtures produced strong indol reactions. Of the other five mixtures, which were all combinations of one *Bact. coli* strain No. 7 with the various *Bact. aerogenes*, 3 produced weak but positive indol tests and two were indol negative. We had learned from the indol sensitivity experiment that the No. 7 *Bact. coli* strain was the slowest indol producer. We therefore repeated the experiment using *Bact. coli* strain No. 7 with 26 *Bact. aerogenes* strains. This time the *Bact. aerogenes* cultures were planted and incubated for twenty-four hours before the No. 7 *Bact. coli* strain in one series and in another series of combinations one loop of the various *Bact. aerogenes* cultures and one loop of No. 7 *Bact. coli* were introduced at the same time. This *Bact. coli* strain failed to produce indol in 8 mixed cultures where the *Bact. aerogenes* strains had a twenty-four-hour longer period of incubation. In the remainder of this series of mixed cultures indol was produced in spite of the twenty-four-hour start of the *Bact. aerogenes* strains. In the series of mixed cultures where this slow indol-producing *Bact. coli* strain was planted at the same time as the various *Bact. aerogenes* strains indol was produced in every case in twenty-four hours. These experiments indicate that in almost all cases of mixtures of *Bact. coli* and *Bact. aerogenes* the presence of the *Bact. coli* strains will be indicated in twenty-four hours by indol formation in tryptophane broth. These experiments also show that indol production is a sensitive index of contamination of a *Bact. aerogenes* culture by *Bact. coli*. In this respect the test is very much superior to the methyl-red test for reasons already discussed.

FAILURE OF BACT. COLI TO PRODUCE INDOL

We believe that some of the reports of failure to produce indol by *Bact. coli* and of indol production by *Bact. aerogenes* are due to the confidence of the observers in colonial characteristics of the organism on isolation media. No doubt, even when the division is based on biochemical characteristics, some members of the *Bact. aerogenes* section produce indol and some members of the *Bact. coli* section do not. However, as shown by Koser

(1923), some soil forms which resemble *Bact. coli* on isolation media and are M.R.+ and V.P.− resemble aerogenes in their ability to utilize citrate and also in their inability to produce indol. It is their carbohydrate metabolism which apparently restricts their colony size and makes them resemble *Bact. coli* on isolation media, but as far as the above tests are concerned there is no more reason for calling them *Bact. coli* than *Bact. aerogenes.* Water analysts, however, on the basis of colony appearance and the M.R. and V.P. tests in the past, no doubt often reported such strains as *Bact. coli* which failed to produce indol. However, Koser has shown that this type is common to soils and rare in animal feces. Brown and Skinner (1930) reported a number of *"B. coli"* isolated from water which utilized citrate. They did not give the indol reactions of these cultures so it is possible that these were also soil forms, similar to those described by Koser, which may be classified as either coli-like or aerogenes-like, depending upon the reactions to which the greatest significance is attached by the observers.

We wish to point out here that among more than 2000 cultures that we have tested with the four tests described, the number that were indol − M.R. + V.P. − and citrate − were rare. We believe that pseudo negative indol tests from fecal *Bact. coli* trains are very seldom obtained.

INDOL PRODUCTION BY COLONY TYPES AND BACT. AEROGENES

If the various types of colonies observed on routine isolation plates are fished and examined for indol production the lack of confidence in the indol test (when isolated colonies are accepted as pure) is easily understood. The results obtained with 686 colonies fished from routine E.M.B. plates from Lake Michigan water samples are shown in table 4. This shows that the incidence of indol production decreased as the colony appearance varied from typical *Bact. coli* to typical *Bact. aerogenes.* If one considers each of these colony types as representative of definite coli-aerogenes strains then of course, indol production is a very variable characteristic of these organisms. We have shown in an earlier section of this paper that many of these colonies are mixtures

of *Bact. coli* and *Bact. aerogenes.* This explains the indol variability in such colonies. Bahlman and Sohn (1924) classified 1223 cultures from routine isolation plates from water samples accord-

TABLE 4

Indol production from various types of colonies on E.M.B. agar

TYPE OF COLONY	TYPICAL COLI WITH SHEEN	COLI TYPE WITHOUT SHEEN	BLUE COLI TYPE	AEROGENES TYPE WITH SHEEN	DOUBTFUL AEROGENES TYPE	AEROGENES POSITIVE TYPE	TYPICAL AEROGENES TYPE	PIN POINT METALLIC SHEEN TYPE	TOTAL ALL TYPES
Number of colonies fished...	181	33	169	29	125	46	95	8	686
Number of colonies producing indol.....	155	22	98	16	37	15	29	1	373
Per cent of colonies producing indol.....	85.7	66.7	58	55.2	29.6	32.6	30.5	12.5	53.4

ing to Standard Methods (1923) on the basis of the methyl-red test, Voges-Proskauer reaction, adonitol fermentation and gelatin liquefaction. Classified by these criteria their cultures gave the following indol reactions:

	CLASSIFICATION			
	Bact. coli	*Bact. aerogenes*	*Bact. cloacae*	Atypical
Number of cultures.....	695	321	20	180
Per cent indol +.....	87	23.4	30	Not given

These cultures were fished from colonies on Endo plates and were not purified before they were differentiated. The percentage indol production found in their *Bact. coli* and *Bact. aerogenes* sections are remarkably close to that found by us for typical *Bact. coli* and typical *Bact. aerogenes* colonies on E.M.B. agar as shown in table 4. Since we know that our colonies were not pure we believe that the variability of the indol reactions in Bahlman and Sohn's cultures may also be explained in part by mixed cultures.

We have shown that the indol test is extremely sensitive and

that the production of indol by indol formers is not easily interfered with, even though they are in the minority, by non indol-producers. We have also found that the ability to produce indol is not easily acquired or lost by a pure strain. These facts support the view that variability in indol production in the *Bact. aerogenes* section may often be due to *Bact. coli* contamination. It is well known that *Bact. cloacae* produces indol. Bahlman and Sohn's data indicate that the incidence of isolation of these strains is low in routine work. We have also found that indol is sometimes formed in *Bact. aerogenes* cultures due to contamination by spore forming organisms. When such cultures are purified, the *Bact. aerogenes* show their normal differential reactions.

In concluding we reaffirm our conviction of the value and importance of the indol test as held by Houston, Hicks, Raghavachari and others. The test requires the shortest incubation period of any of those described. It is simple, positive and very sensitive. The indol once produced is stable in the alkaline tryptophane medium and the reaction is not easily interfered with. Mixtures may therefore be easily indicated and detected with it. The ability to produce indol is not easily acquired or lost. Danger of pseudo negatives with fecal *Bact. coli* strains has been overemphasized. Though some strains of *Bact. aerogenes* sections may produce indol their incidence is low. This test with the other three described may be used in conjunction with colony appearance and the Gram stain report to differentiate the group and to detect mixed cultures which would otherwise be classed as pure.

CONSTANCY OF BIOCHEMICAL REACTIONS

Before discussing the interpretation of results with the four tests described we wish again to stress the fact that these reactions are all very constant in pure cultures. The coli-aerogenes group may form dissociants on agar plates which would not be recognized as belonging to the parent strain. We have found that these dissociants, however, produce the same reaction combination of the aforesaid biochemical tests as the parent strain. Many of our water samples may contain such dissociants and it

seems likely that this sometimes hinders the proper macroscopic differentiation of certain colony types on isolation plates. To show the variation in colony production by *Bact. coli*, the types of colonies produced by the 38 pure strains that were used in some of our experiments are shown below:

B. Coli strains

Lactose+, Indol+, M.R.+, V.P.−, Cit.−

TYPE 1—TYPICAL B. COLI COLONIES ONLY	TYPE 2—TYPICAL B. COLI AND BLUE COLONIES	TYPE 3—BLUE TYPE COLONIES ONLY	TYPE 4—TYPICAL COLI AND DOUBTFUL AERO-GENES TYPE COLONIES	TYPE 5—AERO-GENES SHEEN TYPE COLONIES	TYPE 6—TYPICAL AERO-GENES AND BLUE COLONIES	TYPE 7—DOUBTFUL AERO-GENES TYPE COLONIES	TYPE 8—BLUE AND DOUBTFUL AERO-GENES TYPE COLONIES
10	6	4	11	2	2	1	2

It is certain that strains in types 5, 6, 7 and 8 would not be recognized as *Bact. coli* and a single differential test with the colonial picture would be confusing. When the four differential tests above always give the *Bact. coli* reaction combination, there can be little doubt of the biochemical classification regardless of colonial appearance. We have been able to alter colonial characteristics of pure strains by various treatments but in no case have we been able to alter the biochemical reaction combination obtained with the above described tests. We have found this true for both the *Bact. coli* and *Bact. aerogenes* sections. We have therefore the utmost confidence in the reliability and constancy of these differential tests.

DIFFERENTIATION

During 1928 and 1929, 1488 cultures isolated from water were differentiated using the M.R., V.P. and Koser's citrate tests. Of these, 150 were isolated from the same number of samples from a deep drilled well at Riverside, Illinois. The remainder came from Lake Michigan water in all stages of treatment from raw to chlorinated tap and from points on the lake from Waukegan, Illinois to Gary, Indiana. The majority of these cultures were tested after they had been completely confirmed by standard methods. In some cases the cultures were picked direct from

isolation plates following positive presumptive tests. Simply stating the percentage of positive or negative reactions obtained with each of the tests may be very misleading. We therefore prefer to give the actual combination of reactions obtained with each culture. The results are shown below, classified according to the eight different possible combinations of reactions with these three tests. In this table the order of the reactions given is always from left to right for the methyl-red test, the Voges-Proskauer reaction and Koser's citrate test respectively.

	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6	GROUP 7	GROUP 8
Reaction combinations.....	+−−	+++	+−+	++−	−−+	−++	−+−	−−−
Number of cultures.....	606	66	399	22	153	92	22	128
Possible interpretation.....	*Bact. coli*	These reaction combinations may be interpreted in several different ways				*Bact. aerogenes*	Not in coli-aerogenes group	Not in coli-aerogenes group

These results were rather disappointing. The numbers in group 7 and 8 were surprisingly large. Eliminating these from consideration there remain 1338 cultures which may be considered to belong to the coli-aerogenes group. Of these, 606 or 45 per cent were apparently *Bact. coli* while only 92 or 7 per cent gave the correct tests for *Bact. aerogenes*. No definite interpretation could be made of the remaining cultures. Groups 2 and 3, representing about 35 per cent of the total, may be interpreted as *Bact. coli*, *Bact. aerogenes*, a mixture of both, or as either one with a non-member of the group as a contaminant. These data show that even though three tests were made on routine confirmed *coli*, *aerogenes* cultures a large proportion of the cultures could not be definitely classified.

During 1930 the studies on the indol, methyl-red, Voges-Proskauer and Koser's citrate tests, that have already been described, were made and these tests were applied to 2,093

cultures isolated from water. There are sixteen possible combinations of the above four differential tests as follows:

	REACTION COMBINATION NUMBER															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Indol	−	+	+	+	+	+	−	−	−	−	+	+	+	−	−	−
Methyl red	+	+	+	+	−	−	+	+	−	−	−	+	−	+	−	−
Voges-Proskauer	−	−	−	+	+	−	−	+	+	−	−	+	+	+	+	−
Koser's citrate	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−

Less than 2 per cent of all the cultures were found in the last six classifications shown above. These strains were distributed as follows:

	REACTION COMBINATION NUMBER					
	11	12	13	14	15	16
Reaction combination in following order: Indol, M.R., V.P., citrate	+−−−	+++−	+−+−	−++−	−−+−	−−−−
Number of cultures of the 2,093	7	3	5	3	7	15
Per cent of total cultures	0.33	0.14	0.24	0.14	0.33	0.72

In this table and hereafter in the paper these biochemical differential reactions will be given in the following order Indol, Methyl-red, Voges-Proskauer and Koser's citrate reaction. For instance −+−+ means that the culture was indol negative, methyl red positive, Voges-Proskauer negative and citrate positive.

While the cultures shown in this table were not studied intensively we believe that reaction combinations eleven to fourteen, inclusive, may be caused by mixed strains and that occasionally *Bact. coli* may be present in any of them. Usually, these combinations indicate the absence of all members of the coli-aerogenes group. The last two reaction combinations indicate strains that do not belong to the coli-aerogenes group. Since cultures having these reactions were comparatively rare we shall not consider them further.

The remaining 2,053 cultures as separated according to their

method of isolation and their differential reactions are shown in table 5. The first group of cultures designated −P− included only cultures which were confirmed by the routine procedure of standard methods and had been reported Gram-negative, pure and non-spore-forming. The second group designated + or −M− or sp. included cultures which were completely confirmed by the Standard Methods but were reported as mixtures when Gram-stained smears were examined microscopically. These mixtures frequently contained spores and in some cases Gram-positive organisms. Pure Gram-positive cultures or cultures

TABLE 5

Differential reactions of confirmed coli-aerogenes group cultures isolated from water

DESCRIPTION OF ISOLATED CULTURES	TOTAL CULTURES	REACTION COMBINATIONS									
		1	2	3	4	5	6	7	8	9	10
		−+−−	++−−	++−+	++++	+−++	+−−+	−+−+	−+++	−−++	−−−+
−P−.....	850	15	334	113	63	67	25	71	20	104	38
+ or −M− or sp.....	368	18	98	45	26	11	17	68	20	49	16
Coli-aerogenes type, E.M.B....	541	17	213	30	15	22	12	49	20	127	36
Coli-aerogenes type, cyanide citrate....	294	9	78	17	10	8	3	35	6	99	29
Total.....	2053	59	723	205	114	108	57	223	66	379	119

containing only spore formers were naturally not included in this group. We have studied this group of 368 cultures to obtain a more reliable index of what such cultures might represent. This number of Gram-positive or negative mixed spore or non-spore forming cultures was accumulated from about 2,500 completely confirmed cultures. The 541 cultures from E.M.B. agar plate colonies were very carefully picked from selected E.M.B. plates. These cultures do not represent a true picture of all cultures obtained and confirmed as coli-aerogenes for plates containing non-characteristic colonies were not included in this group. The 294 cultures picked from Noble's ferrocyanide citrate pour plates

were obtained from a smaller number of sampling points and from raw water only. These four groups of cultures should therefore not be compared as representing the same group of water samples. The results by these methods of isolation do show that all of the various combinations of differential reactions are obtained in each case. The percentages of the various differential combinations obtained on these cultures are shown in table 6. It will be noticed in this table that the percentages of the most common *Bact. coli* as indicated by the indol +, M.R. +, V.P. − and citrate − reaction combination and also *Bact. aerogenes* or *Bact. cloacae* as

TABLE 6

Percentage of various differential reaction combinations obtained with coli-aerogenes cultures isolated from water

REACTION COMBINATIONS Indol | M.R. | V.P. | Citrate	−P−	−M−, −Msp.	E.M.B. COLONIES	CYANIDE CITRATE COLONIES	ALL CULTURES
−+−−	1.7	4.9	3.1	3.1	2.9
++−−	39.3	26.7	39.4	26.5	35.2
++−+	13.3	12.2	5.5	5.8	10.0
++++	7.4	7.1	2.8	3.4	5.5
+−++	7.9	3.0	4.1	2.6	5.3
+−−+	2.9	4.6	2.2	1.0	2.8
−+−+	8.4	18.5	9.0	11.9	10.9
−+++	2.3	5.4	3.7	2.0	3.2
−−++	12.3	13.3	23.5	33.7	18.5
−−−+	4.5	4.3	6.7	9.8	5.8

indicated by the indol −, M.R. −, V.P. + and citrate + reaction combination are surprisingly low. It may be pointed out that the percentage of these *Bact. coli* isolated by the routine method from all classes of water was higher than the percentage of these organisms isolated from the ferrocyanide citrate pour plate medium from raw waters only. This is easily understood when it is recalled that the standard method recommends that the most typical colonies be picked. Consequently the *Bact. coli* types are usually picked on routine plates and the *Bact. aerogenes* types are neglected unless the plates only contain such types. In the case of direct planting of samples on ferrocyanide citrate

plates both types are more often found on one of the dilutions of every sample and in all such cases both types were fished.

The total percentage of the common *Bact. coli* and *Bact. aerogenes* types as indicated by the reaction combinations + + − − and − − + + seem to be very low. We have therefore assembled the data from several observers on this point in table 7. This table indicates that Lewis and Pittman (1928) obtained a variation from 39 per cent in water of high sanitary quality to 76

TABLE 7

Percentage of Bact. coli and Bact. aerogenes cultures obtained by biochemical differentiation by various observers

OBSERVER	SOURCE AND TYPE OF WATER	TOTAL COLI-AEROGENES STRAINS ISOLATED	PERCENTAGE THAT DIFFERENTIATED AS REGULAR BACT. COLI AND BACT. AEROGENES
Bahlman and Sohn	Ohio River, raw, settled, filtered and chlorinated	1,223	71.4
Lewis and Pittman	Polluted water	83	76.0
	High sanitary quality raw	196	39.0
Sanitary district of Chicago	Lake Michigan, raw, cyanide citrate cultures	294	60.5
	Selected E.M.B. plate cultures	574	63.0
	Lake Michigan, raw, settled, filtered, and chlorinated, −P−	850	51.5
	Same as above but + or − mixed − or sp.	368	40.0

per cent in polluted water of these common regularly reacting *Bact. coli* and *Bact. aerogenes* strains. Even in water samples originating from the highly polluted Ohio River, Bahlman and Sohn obtain only 71.4 per cent of *Bact. coli* and *Bact. aerogenes* which conformed to these strict definitions. The percentages of such strains that were obtained from Lake Michigan are within the variations obtained by Lewis and Pittman.

Referring to table 5 we will assume for the time that reaction

combinations Nos. 1 and 2 are *Bact. coli* and that 9 and 10 are *Bact. aerogenes*. How are we going to interpret the intermediate reaction combinations of numbers 3 to 8 inclusive? The total percentage of these intermediates found in our groups of cultures were as follows:

GROUP	−P−	+ OR −M− SP.	E.M.B. COLONIES	FERRO-CYANIDE CITRATE COLONIES
Percentage intermediates.....	42.2	50.8	27.3	26.7

This shows that the lowest percentage of these intermediates was found in Noble's ferrocyanide citrate pour plate cultures and the highest in cultures isolated by the standard method but reported mixed after microscopic examination. From this we infer that these intermediate reaction combinations may contain mixtures of strains.

PURIFICATION OF INTERMEDIATE REACTION COMBINATION CULTURES

To study the apparent intermediate strains, 266 such cultures were purified. This group included 28 cultures that gave the − − − + reaction combination. About fifty cultures that were purified originated in the E.M.B. agar colony culture group. Fifty more were cultures that were completely confirmed but were reported as mixed when examined microscopically. The remainder and largest portion were completely confirmed and reported as Gram-negative, pure, non-spore-forming. While it might be desirable to consider the results of purification of each of these sections separately we have summarized them in one group. This was done because the results from all sections were very much alike and also to reduce the number of tables and simplify the presentation.

Figure 3 shows graphically the methods of purification used in the work. The diagrams are almost self-explanatory and need little comment. As many different colonies as were present were picked from the first reisolation plate so that four or five subcultures were usually carried through one of these methods for each

of the original 266 cultures. We believe that the first method is the least satisfactory. Some *Bact. coli* strains were lost by the second method where they were carried through all the media at 46°C. Experience with this method also showed that some *Bact. aerogenes* grew well at 46°C., even on E.M.B. agar plates and in brilliant green broth. The experiments with method two incidentally indicated that Leiter's (1929) report, that implantation of water portions into glucose broth and incubation at 46°C. resulted in an index indicative of only the fecal *Bact. coli* strain

TABLE 8

Result reaction combination after purification of cultures having intermediate differential reaction combinations when isolated

ORIGINAL DIFFERENTIAL REACTION COMBINATIONS	ORIGINAL NUMBER OF CULTURES	BIOCHEMICAL REACTIONS OF STRAINS AFTER PURIFICATION									
		−+−−	++−−	++++	++−+	+−++	+−−+	−+−+	−+++	−−++	−−−+*
++++	47	2	14	8	8	4	1	6	6	17	1
++−+	71	1	24	13	15	5	1	14	6	12	6
+−++	50	1	18	1	3	4	0	5	1	32	2
+−−+	28	0	11	2	0	5	0	1	0	15	1
−+−+	31							24		6	1
−−−+	28							2	1	14	13
−+++	11							2	5	5	
Total	266	4	67	24	26	18	2	54	19	101	24

* Strains having this reaction combination after purification usually do not ferment lactose and do not belong to the coli-aerogenes group.

from warm-blooded animals, is not correct. Method four, utilizing direct planting of the culture to be purified into Noble's ferrocyanide citrate agar has the advantage of requiring the shortest time to obtain the final results. It requires more skill and manipulation time, however, and has other disadvantages. We prefer and recommend method three as being very simple, easy to manipulate, and capable of excellent results and we have adopted it for routine purification studies.

For simplicity the results of the purification have been summarized in tables 8, 9 and 10. Table 8 shows the reactions of the

TABLE 9

Summary of results after purification of cultures having intermediate differential reactions

ORIGINAL DIFFERENTIAL REACTION COMBINATIONS Indol / M.R. / V.P. / Citrate	TOTAL NUMBER CULTURES PURIFIED	BIOCHEMICALLY DIFFERENT STRAINS ISOLATED AND DIFFERENTIATED AFTER PURIFICATION		CULTURES FROM WHICH TWO OR MORE WERE ISOLATED		CULTURES HAVING NO CHANGE IN REACTIONS AFTER PURIFICATION		NUMBER OF STRAINS ISOLATED, NOT BELONGING TO COLI-AEROGENES GROUP	
		Number	Percentage*	Number	Percentage*	Number	Percentage*	Number	Percentage†
++++	47	67	143	19	40	7	15	1	1.5
++−+	71	97	137	24	34	11	15	6	6.2
+−++	50	67	134	15	30	3	6	2	3.0
+−−+	28	35	125	7	25	0	0	1	3.6
−+−+	31	31	100	0	0	24	77	1	3.2
−−−+	28	30	107	2	7.1	11	39	13	43.0
−+++	11	12	109	1	9	4	36	0	0
Total..	266	339	127	68	26	60	23	24	7.1

* Percentage of original cultures.

† Percentage of total strains after purification.

TABLE 10

Classification of intermediate reacting cultures before and after purification

REACTION COMBINATIONS Indol / M.R. / V.P. / Citrate	BEFORE PURIFICATION		AFTER PURIFICATION	
	Number	Percentage	Number	Percentage
−+−−	0	0	4	1.2
++−−	0	0	67	19.8
−−++	0	0	101	29.8
++++	47	17.7	24	7.1
++−+	71	26.6	26	7.6
+−++	50	18.8	18	5.3
+−−+	28	10.5	2	0.6
−+−+	31	11.7	54	15.9
−+++	11	4.2	19	5.6
−−−+	28	10.5	24	7.1
Total.....	266	100	339	100

various strains that were obtained after purification. It fails to show how many cultures were not changed by the purification method. It represents the total resultant picture, however, showing that many of these originally irregular or intermediate reacting cultures conformed to the more common *Bact. coli* and *Bact. aerogenes* reacting strains after purification.

Table 9 shows the increase in the total number of different strains obtained as a result of purification. It indicates that from cultures which gave both positive indol and citrate reactions, two strains were isolated from 25 to 40 per cent of the time depending upon the reaction combination. It shows that except for the $-+-+$ reaction combination the percentage of these cultures which had no change in their differential reactions as a result of purification was low. Table 10 shows the classification and the percentages of the total group of cultures before and after purification. This indicates that, after purification, 50.8 per cent total strains obtained are classified as *Bact. coli* and *Bact. aerogenes* having the characteristic reaction combinations.

DISCUSSION OF RESULTS OF PURIFICATION STUDY

Our data indicate that cultures having the reaction combination $---+$ usually contain *Bact. aerogenes*, a non-member of the group or both. Such cultures must be purified and examined again for proper classification. Occasionally these cultures may contain the $-+-+$ reacting strain.

While comparatively few $-+++$ reacting cultures were studied, three strains were most commonly obtained from them. About half of these cultures showed no change in their reactions by the ordinary purification processes. Pure *Bact. aerogenes* were obtained in four cases and in one case two strains were separated, one of which was *Bact. aerogenes* and the other the $-+-+$ intermediate strain. We also believe that some of these $-+++$ reactions are caused by mixtures of *Bact. aerogenes* and glucose-fermenting organisms which do not belong to the coli-aerogenes group. This reaction combination may also result from *Bact. aerogenes* and *Bact. coli* strains which do not produce indol, or it may result from *Bact. aerogenes* and *Bact. coli* that are weak

indol producers and are outgrown by the *Bact. aerogenes* in the tryptophane broth as was shown in the indol section of this paper. We believe that this combination invariably represents a mixture of strains.

Those cultures having $-+-+$ reaction combinations were found to be apparently pure strains in over 70 per cent of the cases. In six cases regular *Bact. aerogenes* reacting strains were obtained after purification. This change may be considered as due to the elimination of M.R. + contaminants from originally mixed cultures or it may be due to the cause suggested by Koser (1924) when he observed similar changes in organisms isolated from soil. Koser considered such changes in reaction to be the result of slow growing *Bact. aerogenes* strains in which the secondary or alkaline fermentation was speeded as a result of laboratory cultivation. We believe that either of these explanations is tenable in some cases. We conclude, therefore, that while some $-+-+$ cultures contain *Bact. aerogenes* ($--++$) the majority of them remain unchanged. Such strains have been reported isolated from soil by Koser (1924) (1926) and from water by Bardsley (1926) Raghavachari (1926) and Lewis and Pittman (1928). We have found these strains extremely rare in human and animal feces (see table 12). Recently Werkman and Gillen (1931) reported that such Coli-aerogenes intermediates fermented glycerol with the production of trimethylene glycol. Typical *Bact. coli* and *Bact. aerogenes* do not have this property and they, therefore, propose generic recognition of this group under the name Citrobacter.

INTERMEDIATES THAT PRODUCE INDOL AND UTILIZE CITRATE

Purification greatly reduced the number of cultures that produced indol and utilized citrate as a sole source of carbon. Every culture which had the reaction combination $+--+$ was changed by the purification process. We believe that this reaction combination is always indicative of a mixed culture. Table 8 shows that *Bact. coli* was isolated almost as frequently as *Bact. aerogenes*. A culture having $+-++$ reactions was the third most frequent result of purification of the above group.

Cultures having the reaction combination + − + + are ordinarily interpreted as being *Bact. aerogenes* or *Bact. cloacae* which produce indol. When 50 such cultures were purified, eleven of them were found to contain both *Bact. coli* and *Bact. aerogenes*. As table 8 shows, fifty of these cultures yielded 32 *Bact. aerogenes* strains and 18 *Bact. coli* strains. One such culture was also found to contain *Bact. coli* and the − + − + strain described above. Only four cultures or 6 per cent of the strains obtained from this group remained with the same reaction after purification. Another 6 per cent of these cultures were altered in some way by purification so that they produced the other intermediate reactions still to be discussed. We conclude, therefore, that *Bact. aerogenes* or *Bact. cloacae* that produce indol are very rarely encountered in the surface waters of the Chicago area.

The cultures having the reaction combination + + − + may be generally considered as *Bact. coli* which utilize citrate. The − + − + group becomes indistinguishable from this group if the indol test is omitted. Table 5 shows that this was one of the most common intermediate reacting groups found. Table 8 shows that when such cultures were purified strains of *Bact. coli*, *Bact. aerogenes* or the − + − + strains were frequently obtained. About 15 per cent of the strains obtained after purification of this group had the same reactions as at the start and another 20 per cent other intermediate reactions. Our results seem to indicate that pure strains having this reaction may be occasionally encountered in surface waters. We believe that such strains are of soil rather than of fecal origin.

The only intermediate group cultures still to be discussed are those giving positive reactions for all of these tests. Table 5 shows a surprising number of cultures having these reactions when isolated. Such cultures are seldom reported in the literature, probably because all of these tests are not usually made on routine cultures from water. Apparently Lewis and Pittman (1928) did not encounter such cultures in Texas, but Koser (1926) did report an atypical strain isolated from soil which may have been identical with these. Purification of our cultures resulted in the separation of a large percentage of *Bact. coli*, *Bact. aerogenes*

and the intermediate $-+-+$ strains. Some of the cultures having all positive differential tests were not affected by the purification process and some strains, with other intermediate reactions, were obtained.

Our study of intermediate coli-aerogenes group cultures that produce indol and utilize citrate leads us to conclude that such cultures, when isolated by the present standard method from surface water samples, are usually mixtures of two or more strains. From 65 such cultures, two strains and in three cases, three strains were separated. Twenty-five different combinations of strains were obtained. The most frequent combination of strains was the common *Bact. coli* and *Bact. aerogenes.* This combination was separated from 29 of these cultures. In one cases, *Bact. coli, Bact. aerogenes* and the $-+-+$ intermediate strain were separated from one colony.

APPLICATION OF RESULTS OF PURIFICATION EXPERIMENT ON THE ORIGINAL INTERMEDIATE CULTURES

It is unfortunate that each culture listed in the intermediate groups shown in table 5 was not purified. Due to the amount of work involved this could not be done. We are assuming that approximately the same results would have been obtained with the entire group as with the 266 cultures that were purified. Each group of intermediates in table 5 was redistributed in the same way as the same groups in table 8. Since we have concluded that the $+---+$ reaction combination never represents a pure strain, this reaction combination was not included after adjustment for purification. The total results before and after adjustment for purification are shown in table 11. This table shows that purification resulted in an increase of 174 *Bact. coli* strains and an increase of 308 regular *Bact. aerogenes* strains, indicating that about 18 per cent of the total *Bact. coli* strains and about 45 per cent of the total regular *Bact. aerogenes* strains isolated were in mixtures when first purified.

It will be noticed that the percentage of $++-+$ intermediate cultures, the $++++$ atypical cultures and the $+-++$ (*Bact. aerogenes,* producing indol) cultures have been reduced con-

siderably. The + + − + strains resemble *Bact. coli* in most of their characteristics, but it is difficult to determine whether they should be classified with *Bact. coli* or with the intermediate strains.

TABLE 11

Differential reactions of the coli-aerogenes cultures from water before and after purification

	REACTION COMBINATIONS	NUMBER OF CULTURES		PERCENTAGE OF CULTURES		SUGGESTED INTERPRETATION
	Indol | M.R. | V.P. | Citrate	Original	After purification	Original	After purification	
1	− + − −	59	69	2.9	3.2	Coli
2	+ + − −	723	887	35.2	41.3	Coli
1 and 2.....		782	956	38.1	44.5	Total coli
3	+ + − +	205	68	10.0	3.2	Intermediate
4	− + − +	223	261	10.9	12.1	Intermediate
3 and 4.....		428	329	20.9	15.3	Total intermediate
5	+ − − +	57	0	2.8	0	Atypical
6	+ + + +	114	62	5.5	2.9	Atypical
7	+ − + +	108	43	5.3	2.0	Aerogenes-cloacae producing indol
8	− + + +	66	68	3.2	3.2	Aerogenes-cloacae possibly contaminated
9	− − + +	379	687	18.5	32.1	Aerogenes-cloacae
7, 8 and 9.....		553	798	27.0	37.3	Total Aerogenes cloacae
10	− − − +	119	87*	5.8	—	Possibly Aerogenes or cloacae
Total.....		2,053	2,145			Coli-aerogenes group

* These cultures were not *Bact. aerogenes* or *Bact. cloacae* and are therefore not included in the Coli-aerogenes group total after purification.

A study of the coli-aerogenes strains present in feces should assist in the interpretation of these results.

COLI-AEROGENES GROUP IN FECES

While numerous investigators have reported on the abundance of *Bact. coli* and the low percentage of *Bact. aerogenes* present in

feces, they have not used our four tests in differentiating the two strains. It is well known that *Bact. coli* is the predominating organism of feces. Our study of growth rates in lactose broth (see first section of this paper) indicates that under this condition *Bact. aerogenes* will very rarely be isolated by the standard method. This point is very well illustrated by the recent work of Brown and Skinner (1930) who reported that, "only *B. coli* (never *B. aerogenes*) were found in human feces." However, if the fecal suspensions are planted directly into Noble's ferrocyanide citrate agar and a number of every type of colony on the more crowded plates is carefully picked, the coli-aerogenes organisms less frequently found in feces will occasionally be isolated. Another and simpler method that we have used to isolate these minority fecal strains depends upon the more rapid growth of such strains in Koser's citrate solution. Dilutions of the fecal suspensions similar to those ordinarily planted in lactose broth are also planted in Koser's citrate solution. After twenty-four hours incubation at 37°C. the highest dilution with visible growth in the citrate solution and the next highest dilution are streaked on E.M.B. agar plates. Representative colonies of all types are then fished for study. Figure 1 shows E.M.B. plates that were obtained by this method and the standard method from

FIG. 1. CULTURES ISOLATED FROM HUMAN FECES. DIFFERENTIAL REACTIONS OF COLONIES

Plates 1, 2, and 3 streaked from citrate cultures

Plate 1			Plate 2			Plate 3		
Colony number	Type	Reaction	Colony number	Type	Reaction	Colony number	Type	Reaction
1	TC	+−++	1	BC	++−−	1	TC	++−−
2	AS	+−++	2	TC	++−−	2	TA	+−−+
3	A+	−−++	3	A±	−+−+	3	A±	++−−
4	A±	−+−+	4	A+	−+−+			
5	A+	−−++	5	A+	−+−+			
6	A±	−+−+						

Plates 4, 5, and 6 streaked from lactose broth cultures

Plate 4			Plate 5			Plate 6		
Colony number	Type	Reaction	Colony number	Type	Reaction	Colony number	Type	Reaction
1	AS	−+−−	1	TC	++−−	1	AS	++−−
2	A±	++−−	2	TC	++−−	2	AS	++−−
3	A±	++−−	3	BC	++−−	3	AS	++−−
4	BC	++−−						

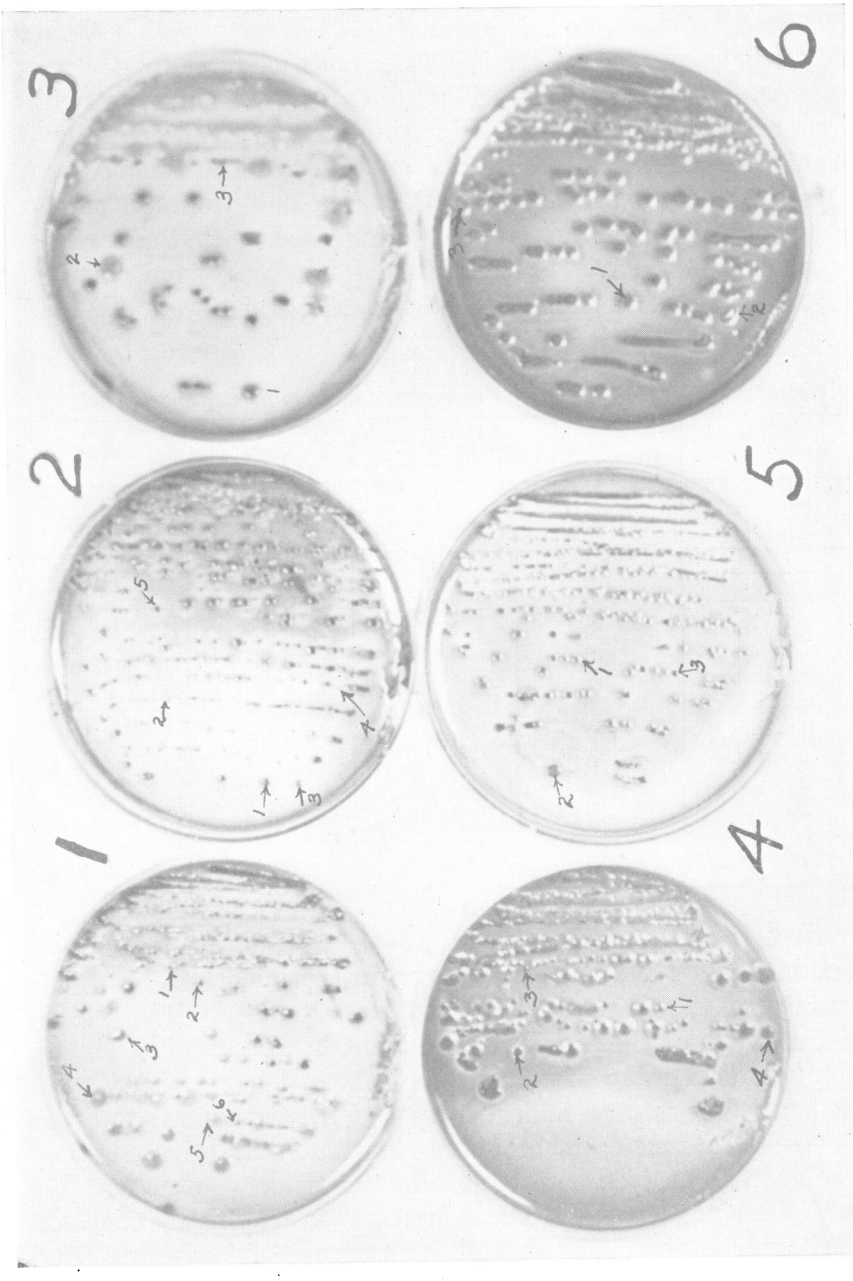

FIG. 1

feces with the colonies that were picked and the differential results obtained. Using this method and Noble's ferrocyanide citrate direct pour plates, we studied 32 samples of feces from eleven persons and ten animals. The animal feces included samples from one guinea pig, two rabbits, three dogs, two sheep and two monkeys. The results obtained on 486 colonies isolated from these samples are shown in table 12. The data from this careful study of a limited number of fecal samples show that apparently all varieties of strains were obtained from the isolated colonies. After purification of all intermediates, all strains were classified

TABLE 12

Differential reactions of the coli-aerogenes cultures from human and animal feces

REACTION COMBINATIONS				NUMBER OF CULTURES		PERCENTAGE OF CULTURES	
Indol	M.R.	V.P.	Citrate	Original	After purification	Original	After purification
−	+	−	−	56	56	11.5	11.5
+	+	−	−	402	406	82.9	83.2
+	+	−	+	3	0	0.6	0
−	+	−	+	9	10	1.8	2.0
+	−	−	+	2	0	0.4	0
+	+	+	+	2	0	0.4	0
+	−	+	+	2	0	0.4	0
−	+	+	+	0	0	0	0
−	−	+	+	8	16	1.6	3.3
−	−	−	+	2	0	0.4	0
Total.....				486	488	100	100

as *Bact. coli*, *Bact. aerogenes* or the − + − + intermediate strain. The last two types were very much in the minority. The percentages of the types given in the tables are not at all indicative of the ratios of these organisms present in feces. They simply apply to this group of cultures as isolated by the methods described. The study indicates that *Bact. aerogenes* and the − + − + intermediates are very rare and that other atypical strains must be even more rare in feces. Since we were not able to find a single + + − + strain in feces we tentatively conclude that such strains isolated from water probably originate from

other sources and therefore should be classified with the − + − + intermediates rather than with *Bact. coli.* It should be pointed out that all of the non-indol-producing *Bact. coli* in this experiment were obtained from one individual. The samples from this person contained no *Bact. aerogenes* or intermediates but contained both types of *Bact. coli* with the non-indol-producing variety making up the great majority of the population. On E.M.B. plates these non-indol-producing *Bact. coli* yielded large aerogenes sheen type colonies and on Noble's ferrocyanide citrate plates they also produced very large colonies resembling *Bact. aerogenes.* The inclusion of three samples of feces from this individual, who apparently is not representative in this respect, probably accounts for the rather high percentage (11.5 per cent) of these strains isolated.

SUMMARY

Preliminary enrichment in lactose broth

1. Our study of preliminary enrichment has indicated that when almost equal numbers of *Bact. coli* and *Bact. aerogenes* are present in water, which is often the case, there is a general tendency for one organism to outgrow the other. It is impossible to predict which strain will gain the ascendancy. The ascendancy of one of the strains depends upon three factors, first, the initial physiological condition of the strains (for this determines the relative length of the lag phase), second, the relative growth rates of the strains in their logarithmic growth phase, third, the relative sensitivity of the strains involved to the products of metabolism. The ascendancy of one of the strains takes place during the first twenty-four hours incubation at 37°C. and the relative numbers of the organisms remain about the same during the second twenty-four hours. The tendency of one organism to overgrow the other is reflected in the E.M.B. agar plate results by Standard Methods on routine samples.

Isolation of coli-aerogenes cultures

1. Levine's eosine-methylene-blue-agar or Skinner and Murray's modification of it appeared to be the best medium for isolation

of the group. Neither of these media is ideal, however. The results obtained with Levines E.M.B. agar are also applicable to other streaked isolation media.

2. Single streaked isolation plates sometimes indicate pure cultures when the cultures are actually mixed. Contaminants of coli-areogenes cultures sometimes mask the colonial characteristics of the coli-aerogenes strains. Many isolated colonies on streaked plates that are considered pure are in reality contaminated with other organisms.

3. Due to the incidence of mixed colonies, the masking effect of contaminants, the variety of colonies produced by pure *Bact. coli*, pure *Bact. aerogenes* and possible dissociants, macroscopic differentiation of routine E.M.B. agar colonies is not successful. It is absolutely impossible to differentiate macroscopically, colonies of the socalled intermediate group strains. Many such strains produce colonies which are similar to typical *Bact. coli* while others are apparently identical with *Bact. aerogenes* in colony appearance (see fig. 2).

4. Microscopic examination of Gram-stained smears does not reveal all coli-aerogenes group cultures that are mixed with other Gram negative non-spore-forming organisms. It never reveals mixtures of *Bact. coli* and *Bact. aerogenes* or mixtures of either of these strans with the intermediate soil forms.

Biochemical differential tests

1. The indol, methyl-red, Voges-Proskauer and Koser's citrate biochemical differential tests were studied with coli-aerogenes strains and with *Bact. coli* and *Bact. aerogenes* mixtures. This study indicated that the methyl-red and Voges-Proskauer tests were the least reliable and were more likely to give misleading or incorrect results due to contaminants. The indol and the citrate tests, on the other hand, are very reliable. Positive tests are practically always obtained when organisms giving these tests are present even though they are initially greatly in the minority. This is not true for the M.R. and V.P. tests. The indol and citrate tests can therefore be used to indicate the possible presence of contaminants. The data indicate that the use of the M.R.

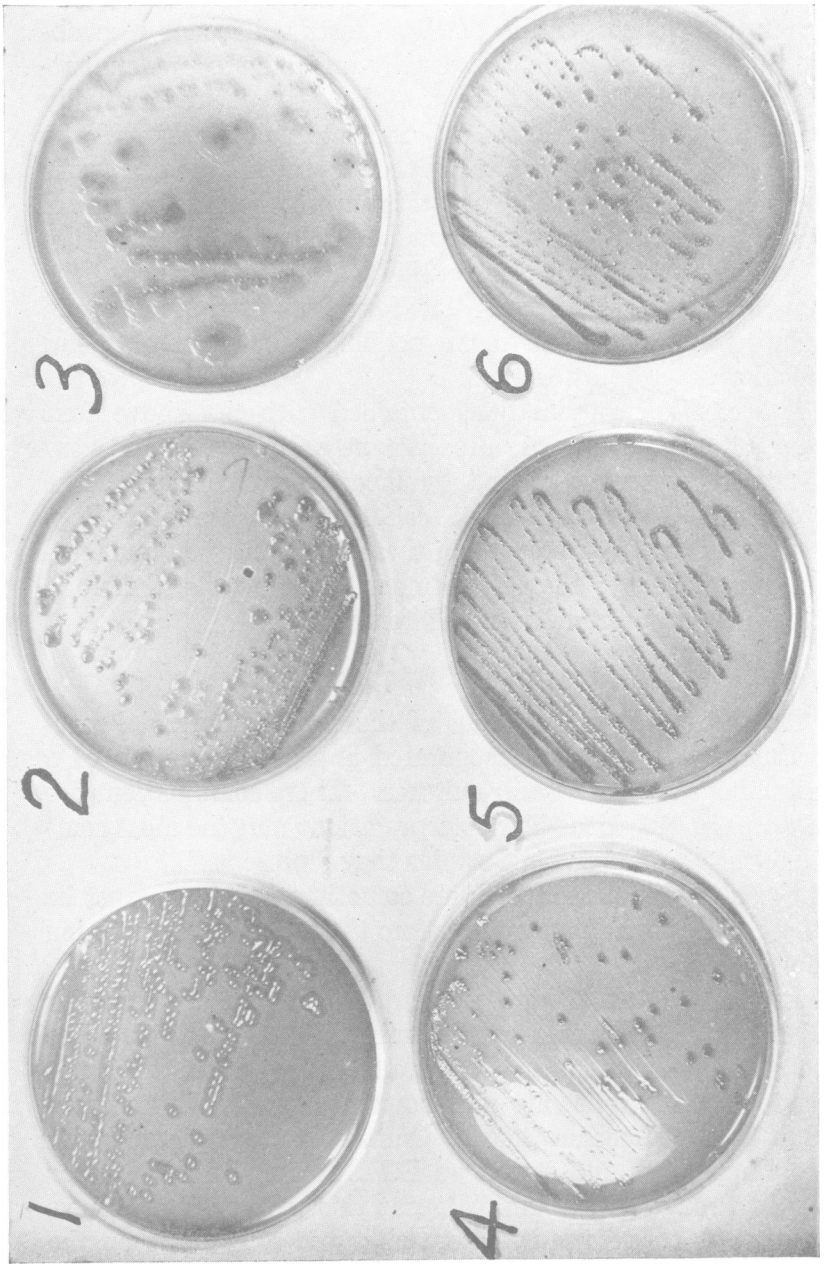

FIG. 2. SHOWING PURE STRAIN COLONIES ON E.M.B. AGAR PLATES

All of these cultures have the following differential reactions: Indol −, M.R. +, V.P. −, citrate +. Plates 1, 2, and 3 have aerogenes colony characteristics. Plates 4, 5, and 6 have coli colony characteristics.

and V.P. tests on routine samples is not satisfactory. Even with mixtures, perfect correlation of these tests will usually be obtained and while the result will usually indicate the predominating organism it will give no indication that the culture is actually a mixture. With these tests intermediate soil strains will be grouped with the fecal *Bact. coli* rather than with the soil forms where they belong. If only two tests are to be used the indol and citrate tests are preferable. We consider the V.P. test the least valuable of the four for routine work where many of the cultures are usually mixtures. The four tests used together are superior to any three and make possible a more accurate and satisfactory interpretation.

2. The reaction combinations obtained with the above four tests on pure *Bact. coli* and pure *Bact. aerogenes* strains are very constant and were not altered by laboratory treatment. The reaction combinations of *Bact. coli,* intermediate and *Bact. aerogenes* strains are different with these tests and they remained different regardless of age or treatment. These tests, therefore, serve as a superior criterion of differentiation.

Differential test interpretation

1. There are ten combinations of the above differential reactions that are commonly encountered in the examination of a large number of surface water cultures. These combinations are listed on page 177 with their interpretations and the most common habitat of the individual strains they represent.

All those combinations which are sometimes mixtures must be purified and differentiated again before the correct interpretation can be made.

2. Several methods of purification of cultures having irregular combinations that indicate possible mixtures were studied. The method of transfer to lactose broth followed by reisolation is not satisfactory. The following method with all subcultures incubated at 37°C. is recommended. Culture in tryptophane broth two to three hours and streak on E.M.B. Incubate plates for twenty to twenty-four hours. Repeat this process three times, each time fishing subcultures from all types of colonies on the

E.M.B. plates. Then fish all subcultures to differential media, lactose broth and agar slants. This method will not purify and yield coli-aerogenes strains from all mixtures in which the coli-aerogenes organisms are present in the minority. In some such instances direct planting in Noble's ferrocyanide citrate agar produces successful coli-aerogenes isolations.

REACTION COMBINATIONS				INTERPRETATION WHEN ISOLATED FROM WATER BY THE STANDARD METHOD		COMMON SOURCE
Indol	M.R.	V.P.	Citrate	Usually	Occasionally	
−	+	−	−	*Bact. coli*	Non-members of group	Feces
+	+	−	−	*Bact. coli*	Very occasionally non-members of group	Feces
+	+	−	+	Mixture of *Bact. coli*, extraneous form or *Bact. aerogenes*	Intermediate strain	Soil
−	+	−	+	Intermediate strain	Mixtures or slow secondary reacting *Bact. aerogenes*	Soil (rarely feces)
+	+	+	+	Mixtures of *B. coli* or *B. aerogenes* with extraneous form	Atypical	Soil
+	−	−	+	Always mixtures, usually contain aerogenes, sometimes coli		
+	−	+	+	Mixtures of aerogenes with extraneous form, sometimes coli	*Bact. cloacae*	Soil
−	+	+	+	Mixtures containing aerogenes	Atypical	Soil
−	−	+	+	*Bact. aerogenes*	Non-lactose fermenting. May or may not be spore forming	Soil (rarely feces)
−	−	−	+	Extraneous form not a member of group	*Bact. aerogenes*	Soil

This study of purification also indicated that all *Bact. coli* strains did not grow and some *Bact. aerogenes* strains did grow at 46°C. on the common laboratory media.

3. A careful study of 32 samples of human and animal feces was made. Two special methods of isolation for obtaining the

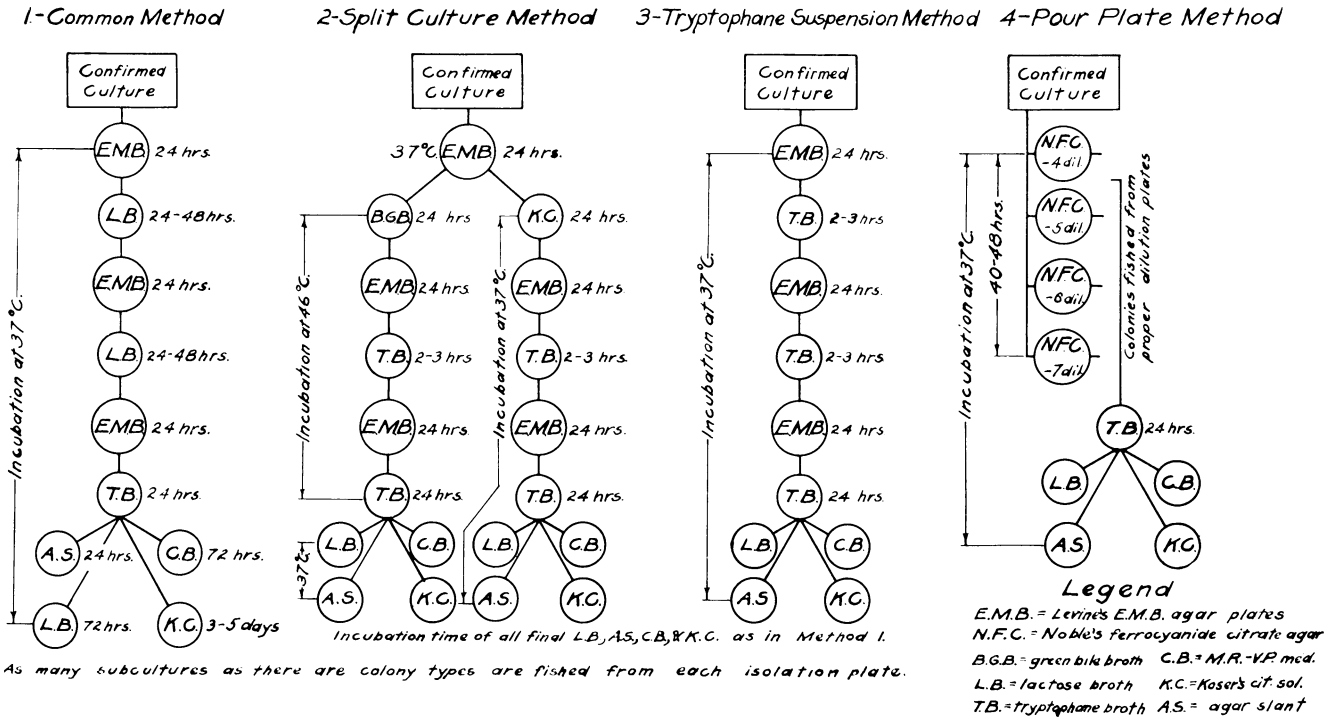
Purification of Coli-Aerogenes Cultures
1-Common Method
2-Split Culture Method
3-Tryptophane Suspension Method
4-Pour Plate Method
Confirmed Culture
E.M.B. 24 hrs.
L.B. 24-48 hrs.
E.M.B. 24 hrs.
L.B. 24-48 hrs.
E.M.B. 24 hrs.
T.B. 24 hrs.
A.S. 24 hrs.
C.B. 72 hrs.
L.B. 72 hrs.
K.C. 3-5 days
Incubation at 37°C.
37°C. E.M.B. 24 hrs.
B.G.B. 24 hrs.
K.C. 24 hrs.
E.M.B. 24 hrs.
T.B. 2-3 hrs.
E.M.B. 24 hrs.
T.B. 24 hrs.
L.B.
C.B.
A.S.
K.C.
Incubation at 46°C.
Incubation at 37°C.
T.B. 2-3 hrs.
E.M.B. 24 hrs.
T.B. 24 hrs.
N.F.C. -4 dil.
N.F.C. -5 dil.
N.F.C. -6 dil.
N.F.C. -7 dil.
Colonies fished from proper dilution plates
40-48 hrs.
Incubation time of all final L.B., A.S., C.B. & K.C. as in Method 1.
As many subcultures as there are colony types are fished from each isolation plate.
Legend
E.M.B. = Levine's E.M.B. agar plates
N.F.C. = Noble's ferrocyanide citrate agar
B.G.B. = green bile broth
C.B. = M.R.-V.P. med.
L.B. = lactose broth
K.C. = Koser's cit. sol.
T.B. = tryptophane broth
A.S. = agar slant

FIG. 3

minority type strains were used. With these methods 462 *Bact. coli* strains, 16 *Bact. aerogenes* and 10 of the − + − + intermediate strains were isolated. None of the other intermediate or atypical strains that may be isolated from water or soil were obtained.

CONCLUSIONS

We conclude from these studies that differentiation of cultures from water obtained after preliminary enrichment in lactose broth is usually unsatisfactory. This is on account of unpredictable elements in the enrichment process, the difficulty of obtaining pure strain isolations and the complexity of the Coli-aerogenes group which requires the use of at least four differential tests for proper interpretation of results. Where exact differential results are necessary, the four tests studied in this paper should be applied to numbers of cultures obtained by direct planting of the samples into some solid medium. Such careful study is very valuable in judging the exact quality of a water when few samples are to be tested.

We also wish to make a plea for more careful study of cultures, including differentiation obtained during studies of the comparative value of various media in routine work. Owing to the manner in which such work is usually conducted at the present time it is impossible to tell the nature of the strains isolated by the standard method, to say nothing of the strains obtained by the new methods or media that are being studied. We believe that, with all of the data now available, improvements might be made in "Standard Methods." Such improvements should be toward an elimination of the deficiencies of the present method without sacrificing the yield of the coli-aerogenes group.

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