DIFFERENTIATION OF THE "INTERMEDIATE" COLI-LIKE BACTERIA

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I. INTRODUCTION

"Intermediate" coli-like bacteria have received much attention since Koser (1923) first reported that *Escherichia coli* did not produce vigorous growth when citrate was present as a sole source of carbon and later (1924) found cultures, similar to *Escherichia coli* in other characteristics, which grew as luxuriantly in the citrate medium as did the *Aerobacter* cultures.

Study of these citrate-utilizing, coli-like bacteria was first confined to confirmation of Koser's work; general recognition of their wide distribution in nature and study of other characters which would differentiate these "Intermediate" forms (Methyl-red positive; Voges-Proskauer negative; citrate positive) from true *Escherichia* (M.R.+; V.-P.−; citrate−) and *Aerobacter* (M.R.−; V.-P.+; citrate+) soon followed.

Bardsley (1926), Raghavachari (1926), Taylor *et al.* (1927), Hill *et al.* (1929), Lewis and Pittman (1928), Holwerda (1928, 1930), Minkewitsch *et al.* (1928), Minkewitsch (1930), Brown and Skinner (1930), Kline (1930), Poe (1931), Pawan (1931), Ruchhoft *et al.* (1931) and Burke-Gaffney (1932) were among those who soon confirmed and extended Koser's work.

Braak (1928) made the first study of glycerol metabolism of "Intermediate" coliform bacteria and reported that such cultures converted glycerol into trimethylene glycol whereas control strains of the genera *Escherichia* and *Aerobacter* did not form the glycol. Werkman and Gillen (1932) confirmed the work of Braak and created the genus *Citrobacter* to include such intermediate strains.

Differential tests, in order to be completely useful, should be rapid, simple and easily executed. In the absence of such a test to determine the production of trimethylene glycol from glycerol, workers continued to use the methyl-red test, the Voges-Proskauer reaction and utilization of citrate for differentiation of the "Intermediate" coliform bacteria.

Fundamental physiological differences in the metabolism of glucose by the coliform bacteria have been recognized since Harden and his associates (1901, 1905, 1905–1906, 1911, 1911–1912) first showed that strains of *Aerobacter* decomposed glucose with the production of acetylmethylcarbinol and 2–3, butylene glycol as well as two or more volumes of CO_2 to one of H_2 and considerably less acetic, lactic and succinic acid, than *Escherichia coli* which did not form appreciable quantities of acetylmethylcarbinol or 2–3, butylene glycol, produced CO_2 and H_2 in a ratio of 1:1 and formed significantly larger quantities of the acids. Furthermore, cultures of *Aerobacter* produced much more ethyl alcohol

than acetic acid from glucose, whereas cultures of *Escherichia* formed these compounds in approximately equimolar quantities. Scheffer (1928) studied the glucose metabolism of the coliform "Intermediate" group and found their metabolic end-products similar to those of *Escherichia* as is shown in table 1.

The Voges-Proskauer reaction, a qualitative test for acetylmethylcarbinol and the methyl red test, a qualitative measure of the intensity and fate of acids produced, are therefore of most value for rapid primary differentiation of the coliform bacteria. The value of these tests, first shown by Levine (1916) to

TABLE 1

End-products of glucose metabolism by coliform bacteria (data from Scheffer, 1928)

PRODUCTS	ESCHERICHIA COLI		"INTERMEDIATE"		AEROBACTER AEROGENES	
	mM.	Per cent of sugar fermented	mM.	Per cent of sugar fermented	mM.	Per cent of sugar fermented
Carbon dioxide	42.3	11.1	31.8	10.9	338.0	39.3
Hydrogen	41.0	0.5	32.5	0.5	100.0	0.5
Lactic acid	78.1	41.9	59.8	41.8	23.6	5.6
Succinic acid	25.4	17.3	21.9	20.1	6.8	2.1
Formic acid	1.96	0.5		0	15.7	1.9
Acetic acid	41.8	14.9	25.8	12.1	3.5	0.5
Ethanol	41.5	11.4	26.1	9.3	139.5	17.0
Acetylmethyl carbinol		0		0	trace	0
2-3 butylene glycol		0		0	110.8	26.4
Total		97.6		94.7		93.3
Ratio CO_2/H_2	1.03:1.00		0.98:1.00		3.38:1.00	

correlate with differences in glucose metabolism, has been firmly established. Koser's citrate test is then used for further separation of the coliform bacteria into three groups:

TEST	V.-P.	M.R.	CITRATE
Escherichia	−	+	−
"Intermediate"	−	+	+
Aerobacter	+	−	+

Unfortunately, earlier investigation of the coliform bacteria was materially hindered by lack of a sensitive qualitative method for determination of the Voges-Proskauer reaction. Thus, some of the cultures diagnosed as representatives of the "Intermediate" section, in reality, belonged to the genus *Aerobacter*. That such was the case was clearly shown by Vaughn, Mitchell and Levine (1939). After finding that some cultures classified as belonging to the "Intermediate" section were really *Aerobacter* strains, it was anticipated that further investigation would show that *Aerobacter* cultures could form trimethylene glycol from glycerol. This was proven by Mickelson and Werkman (1940).

Most confusion in classifying the "Intermediate" group has resulted from a tendency to allocate *Aerobacter* cultures having very weak or negative Voges-Proskauer reactions to the "Intermediate" section without realizing that, of the two compounds, acetylmethylcarbinol and 2–3, butylene glycol, the latter predominates and the carbinol is sometimes found only in traces, even when estimated quantitatively. Also, acetylmethylcarbinol is decomposed by the coliform bacteria according to Linton, (1925); Paine, (1927); Williams and Morrow, (1928); and Tittsler, (1938). Furthermore, routine use of an incubation temperature of 37°C. for the coliform bacteria mitigates against detection of *Aerobacter* when using the Voges-Proskauer reaction as a criterion, as does too short or too long incubation. Further confusion has resulted from failure to recognize that the citrate test is a measure of the ability of the coliform bacteria to readily utilize the citrate radical as *a sole source of carbon in an otherwise inorganic medium.*

In a search for other characteristics which would be more satisfactory for separating the "Intermediate" group from true *Escherichia* and *Aerobacter*, several differential tests have been suggested including the production of hydrogen sulfide, utilization of cellobiose and indole formation in tryptophane broth.

Levine and his students, (1932) found that the "Intermediate" cultures investigated by them formed hydrogen sulfide in proteose peptone, ferric citrate agar and later (1934) reported that not all "Intermediate" cultures produced hydrogen sulfide in their medium. Finally, Vaughn and Levine, (1936) showed that the diagnostic value of the proteose peptone, ferric citrate agar medium for hydrogen sulfide production was dependent upon the concentration of agar contained in the medium. A concentration of 1.5 per cent agar was optimum for differential purposes. With higher concentrations of agar, the number of positive cultures was materially reduced whereas, in a medium containing appreciably smaller amounts of agar the differential value was completely lost.

It is to be emphasized that hydrogen sulfide production is constant only for a constant set of conditions. Experience has shown that proteose peptone ferric-citrate medium with 1.5 per cent agar is the most satisfactory medium for use as a differential test for the "Intermediate" coli-like bacteria. Media with added sulphur-containing compounds or employing more sensitive indicators are as inadequate for good differentiation as liquid proteose peptone ferric-citrate medium in which it has been shown that the majority of strains of *Escherichia* and *Aerobacter* as well as the "Intermediate" coli-like cultures are capable of producing H_2S from the sulphur-containing constituents of the peptone.

The fermentation of cellobiose was first used by Jones and Wise (1926) and Koser (1926) with later confirmation by Skinner and Brudnoy (1932) and more recently by Tittsler and Sandholzer (1935), Stuart *et al.* (1938) and Mitchell and Levine (1938). Cellobiose fermentation will distinguish between true *Escherichia* and "Intermediate" cultures but it must be remembered that *Aerobacter* cultures attack cellobiose.

Other tests, particularly indole formation from tryptophane broth, have been used in an attempt to separate the coliform bacteria. Here, too, success has

been limited because of the high percentage of *Aerobacter* cultures which produce indole. Indole is a character better suited to specific than to generic differentiation.

As a result of the confusing and ambiguous results obtained with the differential tests cited above, some workers believed that part of the "Intermediate" organisms should be allocated to *Escherichia* and the rest to *Aerobacter* (Carpenter and Fulton, 1937). Others considered them all as *Escherichia* (Tittsler and Sandholzer, 1935; Yale, 1939); still others refused to allocate them but retained the "Intermediate" designation (Koser, 1924; Ruchhoft *et al.*, 1931; Bardsley, 1934; and many others); some gave them separate generic rank as *Citrobacter* (Werkman and Gillen, 1932; Bartram and Black, 1937) and some placed all of the coliform bacteria in one genus, *Bacterium* (Minkewitsch, 1930; Skinner and Brudnoy, 1932; Parr, 1938; and Malcolm, 1938).

Regardless of the manner in which the taxonomy of the group is finally treated, the "Intermediate" coliform bacteria are widely recognized and with more experience and more refined diagnostic methods, two alternatives are apparent: the "Intermediate" coliform bacteria must either be recognized as a separate genus or be given specific allocation in some existing genus. In either case, more attention must be given to specific and varietal differences.

The following information is presented with respect to the taxonomic status of the "Intermediate" coli-like bacteria; to differentiate them from *Aerobacter* and the characteristic strains of true *Escherichia* and to offer satisfactory species and varietal descriptions.

II. CHARACTERISTICS OF THE "INTERMEDIATE" COLI-LIKE BACTERIA

A. Source of cultures

The cultures to be considered are true "Intermediate" coli-like bacteria: gram-negative, short rods; do not produce spores; are generally motile with peritrichous flagellation; ferment lactose with acid and gas; do not produce acetylmethylcarbinol (V.-P. negative); and utilize citrates as a sole source of carbon (citrate positive).

The 223 cultures included in this investigation were obtained from a wide variety of sources including the feces of humans and fowls, various types of waters and soils, chicken eggs, milk, oysters and olives. The collection included cultures from laboratories in various parts of the world as well as strains isolated by the authors.

B. Methods

The cultures were all subjected to serial replating on Levine's eosine-methylene-blue agar for purification. Well isolated colonies were picked and purified by repeated plating from lactose broth (Standard Methods, 1936).

Primary differentiation from true *Escherichia* and *Aerobacter* was made on the basis of the Voges-Proskauer and Koser's citrate tests. The V.-P. reaction was determined by the use of Barritt's (1936) alpha-naphthol reagents on cultures grown in Difco M.R.-V.P. medium at 30°C. for periods of from one to

five days, (Vaughn, Mitchell and Levine, 1939). One ml. of culture was used to which was added 0.6 ml. of 5 per cent α-naphthol in *absolute ethyl alcohol* and 0.2 ml. of 40 per cent KOH. The V.-P. reactions, using Barritt's reagents, are best observed 30 minutes to 6 hours after addition of the reagents. On long standing, the crimson to ruby color denoting a positive reaction becomes darkened to brownish color which may turn very dark brown and form a heavy precipitate or return to a coppery color characteristic of a negative reaction.

The ability of the cultures to utilize the citrate radical as the sole source of carbon was determined by use of *Difco* Koser's citrate medium. Care was taken to exclude extraneous carbonaceous and nitrogenous contamination by acid washing and carefully rinsing all test tubes with distilled water. The medium was prepared with distilled water. Inoculations were made from 18- to 24-hour nutrient broth cultures using a straight needle.

Secondary or specific allocation, using the production of hydrogen sulfide from proteose peptone ferric citrate agar, the formation of indole in tryptophane broth, using Kovac's reagent (1928), the fermentation of starch, aesculin, salicin and glycerol followed the suggestion of Levine *et al.* (1934). These and other carbon compounds were added in concentrations of 0.3 to 0.5 per cent to a basal medium containing 5 grams of Bacto-peptone, 1 gram of K_2HPO_4 and 10 ml. of Andrade indicator per liter of distilled water.

The starch and aesculin media were prepared differently. Since it is important that the starch used be free from reducing substances, *Kingsford* and *Argo* corn starches known not to reduce Fehling's solution were used. A concentration of 1 per cent starch was prepared by making a thin paste in a small portion of cold basal medium and adding this paste to the desired quantity of boiling basal medium. With care a satisfactory suspension of the starch is obtained. *Soluble starches are unsatisfactory for differential purposes as most coliform bacteria readily ferment them*, since they contain products of starch hydrolysis.

The aesculin medium was prepared by adding 0.3 per cent aesculin and 0.05 per cent ferric citrate to the basal medium. Decomposition of the aesculin is denoted by blackening of the medium and accumulation of gas in the Durham tubes.

Motility was determined by hanging drop preparations of young cultures grown in nutrient broth and by the use of semi-solid agar (nutrient broth plus 0.5 per cent agar). Flagella stains, where made, were prepared according to the method of Plimmer and Paine (1921).

C. Differentiation of the "Intermediate" section

When it was recognized that an "Intermediate" group of coli-like organisms existed, primary differentiation from true *Escherichia* and *Aerobacter* cultures was made by the use of the Voges-Proskauer, methyl-red and Koser's citrate tests. Soon, however, *Aerobacter*-like cultures were isolated and classified as "Intermediate" coli-like bacteria and confusion reached such a state that some workers felt it would be desirable to allocate all "Intermediate" cultures to the two existing genera, depending upon which genus was more closely simulated.

As pointed out previously, part of the confusion arose through inadequate control of differential criteria. The fundamental differences in glucose metabolism were not widely recognized. Furthermore, a tendency to attempt to use

TABLE 2

Differentiation of sections of the coliform bacteria

CHARACTER	ESCHERICHIA	"INTERMEDIATE"	AEROBACTER
V.-P.	−	−	+
Citrate	−	+	+
M. R.	+	+	−
H_2S	−	+	−
Cellobiose	−	+	+
Uric acid (1)	−	−	+
Urea (1)	−	+	+
Yeast Nucleic acid (1)	−	−	+
Allantoin (1)	−	−	+
Hydantoin (1)	−	−	+
Uracil (1)	+	−	+
Growth at 45°–46°C. (2)	+	−	−
Resistance to H_3BO_3 (3)	+	−	−
Decomposition of Na malonate (4)	−	−	+
Metabolite specificity (5)	Specific for *Escherichia*	Specific for "Intermediate"	Specific for *Aerobacter*
Bacteriophage specificity (6)	Specific for *Escherichia*	Specific for "Intermediate"	Specific for *Aerobacter*
Origin	Predominant in vertebrate feces	Indefinite	Predominant in non-fecal materials

(1) Mitchell and Levine, (1938); West, Gililland and Vaughn (1941)
(2) Levine, Epstein and Vaughn (1934)
(3) Levine, (1921); Levine, Epstein and Vaughn, (1934); Vaughn and Levine, (1935); Vaughn, (1935)
(4) Leifson, (1933); West, Gililland and Vaughn (1941)
(5) Powers and Levine (1937)
(6) Powers, (1938); Powers, Levine and McCleskey (1938)

characters for generic diagnosis which are most useful for species or varietal differentiation still exists. What, then, are the limits of group differentiation?

The characters shown in table 2 appear particularly satisfactory for group differentiation.

On the basis of the differences shown in the table it is felt that there is sufficient evidence for regarding the true "Intermediate" coliform bacteria as a separate, distinct group. It is true that in some characteristics the "Intermediate" group appears more closely related to *Escherichia* as shown by the metabolic end-products of glucose fermentation as manifested by the negative Voges-Proskauer reaction and the positive methyl-red test for true *Escherichia*. On the other hand, characteristics suggestive of *Aerobacter* include the utilization of citrate as a sole source of carbon, failure to grow at 45–46°C., and low resistance to boric acid media.

The most outstanding characteristic possessed by the "Intermediate" section not common to *Escherichia* or *Aerobacter*, is the ability to form hydrogen sulfide from proteose peptone ferric-citrate agar.

Generic ranking of the "Intermediate" section has presented a confusing problem in the past. Purely on the basis of glucose metabolism the "Intermediate" bacteria are closely related to true *Escherichia* as has already been stressed. The genus *Citrobacter* as originally created for the "Intermediate" section was not satisfactory for it permitted the presence of *Aerobacter* types—"*acetoin not produced from glycerol and rarely from glucose and then only in traces.*" Increase in our knowledge of the coliform bacteria and the advent of more refined techniques have, however, shown that the "Intermediate" group can be differentiated. Furthermore, the section has been widely recognized and specifically differentiated from *Escherichia* and *Aerobacter*. It would seem that steps should be taken to insure a legitimate taxonomic position for the "Intermediate" coliform group.

If the term *Citrobacter* is to be accorded generic rank, then the characterization by Mitchell and Levine (1938) should be given serious consideration. They point out as shown in table 2 that the "Intermediate" coli-like bacteria (*Citrobacter*) differ from true *Escherichia* cultures in that the former (1) utilize urea but not uracil as a sole source of nitrogen, (2) utilize citric acid and cellobiose as sole carbon sources, and (3) generally produce H_2S but not indole. Further evidence to strengthen the position of the "Intermediate" coli-like bacteria is also to be found in table 2 where it has been shown that the "Intermediate" coli-like bacteria differ from true *Escherichia coli* in that they (1) do not grow well at 45°–46°C. (Eijkman test), (2) are not resistant to 0.325 per cent boric acid in buffered lactose broth, (3) produce "metabolites" which inhibit a large proportion of intermediate strains but do not appreciably influence the growth of *Escherichia* or *Aerobacter* cultures, (4) exhibit marked group specificity for bacteriophages ("Intermediate" phages attack only "Intermediate" cultures), and (5) do not have a definite origin but are apparently quite evenly distributed in a wide variety of habitats (see table 6).

The genus *Citrobacter* (Werkman and Gillen) has not been satisfactory for allocation of the "Intermediate" coli-like bacteria. The physiological similarity between "Intermediate" coli-like cultures and true *Escherichia coli* cultures as indicated by the metabolism of glucose might be considered of sufficient magnitude to make the allocation of the "Intermediate" section to the genus *Es-*

cherichia mandatory. Such allocation, however, should be made with the understanding that the presence of "Intermediate" coli-like cultures in a food (as for example oysters or olives) is not to be regarded as *prima facie* evidence of fecal pollution. From the standpoint of sanitary significance, allocation of the "Intermediate" section to the genus *Escherichia* can not be completely satisfactory until the origin of the "Intermediate" coli-like organisms is conclusively proved. Evidence available at present does not indicate a clear cut solution of the question of the sanitary significance of "Intermediate" coli-like bacteria found in foods or in water.

The "Intermediate" coli-like bacteria possess the following general characteristics:

Gram-negative short rods; do not form spores; generally motile—if motile, with peritrichous flagellation; ferment lactose with production of acid and gas; ferment glucose with formation of equal volumes of CO_2 and H_2 but no acetylmethylcarbinol (V.-P. negative) and form significant quantities of lactic, acetic and succinic acids (M.R.+); utilize citric acid as a sole source of carbon; generally produce H_2S in proteose peptone ferric-citrate agar; decompose cellobiose with the production of acid or acid and gas; utilize urea as a sole source of nitrogen but not yeast nucleic acid, uracil, uric acid, allantoin or hydantoin; do not grow well at 45°–46°C. (Eijkman test negative); fail to grow in boric-acid lactose broth at 42°–43°C.; have an optimum temperature range of 30° to 37°C.

It is to be emphasized that with most of these characters, at least a few cultures in any of the three sections will be found to be in disagreement with the majority. Other characters, such as indole production, are not considered of significance for generic differentiation although very useful for species identification. (A large number of *Aerobacter* cultures produce indole.) All are conversant with the vagaries of the methyl-red reaction, and since when properly ascertained it correlates so well with the V.-P. reaction its use for differentiation is not pertinent.

D. Specific differentiation

Results of investigation on the carbon compounds decomposed by the "Intermediate" cultures is summarized in table 3.

Of the 27 compounds tested, 14 were attacked by all, or nearly all cultures, only 2 were not decomposed and 12 were considered of possible value for species differentiation.

It has already been shown that hydrogen sulfide production is a characteristic common to most of the "Intermediate" section. Experience has shown that this character correlates well with other reactions for the purposes of differentiating specific types of "Intermediate" bacteria.

Table 3 shows the specific differences between the H_2S positive and negative cultures. One hundred ninety-six cultures produced H_2S and only 27 cultures were negative. Of the 12 characteristics used in segregation of these two groups only a few were of significant differential value. The other carbon compounds decomposed were attacked at random by both groups and an attempt to em-

ploy all such characters would tend to increase the number of groups geometrically by the expression 2n where "n" is the number of characters studied. Obviously, such a practice would greatly confuse satisfactory grouping of the cultures.

Those characters thought to be of value for differentiation of the H_2S positive and negative types included indole production, motility, and fermentation

TABLE 3

Group differentiation of the 223 "Intermediate" Coli-like cultures

GROUP	ALL STRAINS (223 CULTURES)		H_2S POSITIVE (196 CULTURES)		H_2S NEGATIVE (27 CULTURES)	
Character*	Positive reactions					
	Number	Per cent	Number	Per cent	Number	Per cent
Indole	17	7.6	3	1.5	14	59.9
Motility	217	97.3	196	100.0	21	77.8
Starch	6	2.7	0	0.0	6	22.2
Aesculin	17	7.6	1	0.5	16	59.2
Salicin	43	19.3	24	12.2	19	70.4
Inositol	5	2.2	0	0.0	6	23.1
Glycerol	216	96.9	195	99.5	21	77.8
Sucrose	127	57.0	112	57.2	14	51.9
Raffinose	125	56.1	112	57.2	13	48.2
Melezitose	21	9.4	21	10.7	0	0.0
Dulcitol	94	42.2	88	44.9	6	22.2
Adonitol	4	17.9	0	0.0	4	14.8
α-methyl glucoside	86	38.6	75	38.3	11	40.8
Na-malonate	20	8.8	11	5.6	9	33.3
Trehalose	222	99.6	195	99.5	27	100.0
Sorbitol	220	98.7	195	99.5	25	92.6
Cellobiose	215	96.4	191	97.5	24	88.9

* Xylose, arabinose, rhamnose, glucose, galactose, fructose, mannose, lactose, maltose and mannitol attacked with acid or acid and gas production by all cultures. Amygdalin and erythritol not decomposed.

of starch, aesculin, salicin, inositol and glycerol. The coefficients of correlation[1] of these characters are shown in table 4.

The coefficients of correlation were calculated by formula I.

$$\frac{a(a + b + c + d) - (a + c)(a + b)}{\sqrt{(a + c)(b + d)(a + b)(c + d)}} \quad (I)$$

as suggested by Yule for situations where perfect correlations of 1 or -1 would result if any one group, a, b, c or d was zero. Otherwise, coefficients of corre-

[1] For a detailed discussion of this method consult Levine (1918).

lation for these characters may be erroneous in that perfect correlations of 1 or -1 are obtained for a large number of characters of little or no significance if the formula (II) $\frac{ad - bc}{ad + bc}$ is used.

For this study, using formula I, it has been considered that if the coefficient of correlation between two characters is greater than 0.5 they may be regarded as correlated, but if less than 0.3 there is probably no correlation.

Inspection of table 4 shows that H_2S production is significantly correlated with indole production and the decomposition of aesculin and to a lesser extent with motility and decomposition of inositol and salicin; the association being negative with the exception of motility.

All of the characters do not correlate with each other. Starch, which does not correlate well with H_2S has a high degree of association with motility and inositol but less significantly with aesculin. Indole production correlates well only with H_2S and aesculin. Glycerol is not correlated with any of the

TABLE 4

*Coefficients of correlation for each pair of significant characters using formula I**

	H_2S	INDOLE	STARCH	AESCULIN	SALICIN	INOSITOL	GLYCEROL	MOTILITY
H_2S		−0.62	−0.14	−0.83	−0.47	−0.45	−0.40	+0.50
Indole	−0.62		+0.16	+0.62	+0.42	+0.06	−0.09	−0.16
Starch	−0.14	+0.16		+0.58	+0.34	+0.83	+0.01	−0.83
Aesculin	−0.83	+0.62	+0.58		+0.60	+0.48	−0.14	+0.58
Salicin	−0.47	+0.42	+0.34	+0.60		+0.33	−0.04	−0.34
Inositol	−0.45	+0.06	+0.83	+0.48	+0.34		+0.03	−0.65
Glycerol	−0.40	−0.09	+0.01	−0.14	−0.04	+0.03		−0.03
Motility	+0.50	−0.16	−0.83	+0.58	−0.34	−0.65	−0.03	

* No significant correlation was found for the other characters.

other characters. Aesculin shows a significant degree of association with all characters except glycerol but the number of positive reactions is very small. Motility correlates with starch, inositol, aesculin and H_2S. Inositol correlates particularly well with starch and motility and to a lesser degree with aesculin and H_2S. Salicin correlates only with aesculin and H_2S although the degree of association is not good.

Two specific groups are evidently represented by the 223 "Intermediate" cultures; a large H_2S positive group represented by 196 cultures and a small H_2S negative group of 27 cultures. The H_2S positive group is well defined. It rarely produces indole; does not ferment starch, aesculin or inositol; is motile and ferments glycerol.

The H_2S negative group is quite heterogeneous. Inspection of the results shown in table 3 indicated that two types or varieties might be present among the H_2S negative cultures. This supposition was tested by determining the coefficients of correlation among the same characters as used for group differentiation. The coefficients of correlation are shown in table 5. There is a high

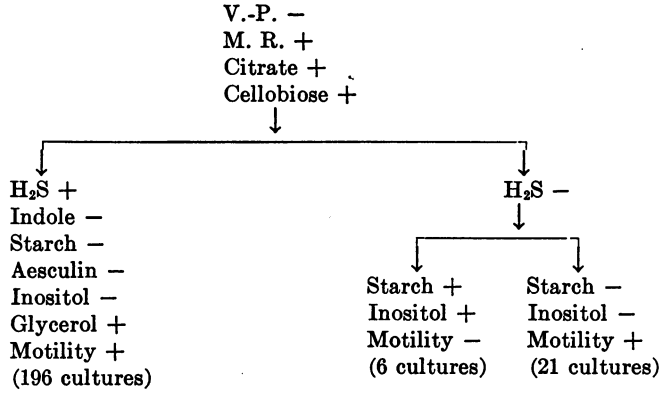
degree of association between starch, inositol and motility. The other characters do not correlate well with the exception of aesculin with salicin and to a lesser extent indole with salicin. Two types are present; one, represented by 6 cultures, ferments starch and inositol but is non-motile; the other, comprising 21 cultures, does not ferment starch or inositol but is motile. (Malcolm, 1938, has also found inositol an aid for specific differentiation of the "Intermediate" coli-like bacteria as indicated by his correlation studies.)

TABLE 5

Coefficient of correlation for each pair of significant characters of the H_2S negative cultures, using formula I

	INDOLE	STARCH	AESCULIN	SALICIN	INOSITOL	GLYCEROL	MOTILITY
Indole		−0.20	+0.40	+0.51	−0.38	+0.38	+0.20
Starch	−0.20		+0.44	+0.35	+0.78	+0.29	−0.79
Aesculin	+0.40	+0.44		+0.78	+0.26	+0.21	−0.44
Salicin	+0.51	+0.35	+0.78		+0.58	+0.47	−0.35
Inositol	−0.38	+0.78	+0.26	+0.58		+0.28	−0.57
Glycerol	+0.38	+0.29	+0.21	+0.47	+0.28		−0.29
Motility	+0.20	−0.79	−0.44	−0.35	−0.57	−0.29	

The "Intermediate" section may be divided into three well defined groups as follows:



Two sections should be recognized among the "Intermediate" coli-like bacteria, an H_2S positive group which is well defined and an H_2S negative group which may be further satisfactorily differentiated. In some other collection of cultures the H_2S negative group might be the larger. Representatives of both groups have been described by many investigators. The two groups should be allocated to the genus *Escherichia.*

As already pointed out, the ability of the "Intermediate" coli-like bacteria to produce hydrogen sulfide in proteose peptone ferric-citrate agar is a characteristic distinctive for most of the cultures of this section of coliform bacteria. For adequate differentiation it is desirable to recognize at least two definite

groups because of the distinct differences between the hydrogen sulfide positive and negative cultures. The large H_2S+group of "Intermediate" coli-like bacteria might be recognized as *Escherichia freundii* (Braak) Yale. The small H_2S−group might be recognized as *Escherichia intermedium* (Werkman and Gillen) *nov. comb.*

Description of Escherichia intermedium. (Werkman and Gillen) nov. comb.

Morphology (at 30°).

Form and arrangement:—Short rods with rounded ends which occur singly, in pairs and short chains when young nutrient agar or broth cultures are examined.

Spore formation:—No spores have been observed.

Motility:—Actively motile with peritrichous flagella or non-motile.

Staining reactions:—Gram-negative.

Cultural characteristics (at 30°C)

Nutrient agar:—Growth is abundant, raised, greyish white with smooth to wrinkled surface and butyrous consistency.

Nutrient broth:—An abundant turbidity is formed with surface growth confined to slight ring formation.

Gelatin stab (at 20°C):—Growth along the line of inoculation but no liquefaction after 60 days.

Potato:—Growth on potato wedges is abundant with a white to ivory color.

Litmus milk:—The milk is acidified; sometimes followed by coagulation and reduction. Gas is formed. Proteolysis has not been observed.

Levine's eosine-methylene-blue agar:—Well isolated colonies vary from 1 to 4 mm in size. There is no confluence of neighboring colonies so characteristic for cultures of *Aerobacter*. Colonies are slightly to moderately raised with surfaces varying from flat to convex and usually smooth and glistening but sometimes dull, rough and granular.

By transmitted light two types of colonies have been observed: 1) Colonies having almost the same appearance throughout but with a distinctly lighter center, the color being similar to the medium. 2) Colonies having a dark brownish central area which diffuses out to a lighter margin.

By reflected light three types of colonies have been observed: 1) Dark, button-like, concentrically ringed colonies possessing a strong greenish metallic sheen so characteristic for *Escherichia coli*. 2) Colonies with dark, purplish, wine-colored centers surrounded by a light pink zone. Some colonies are concentrically ringed. 3) Pink colored colonies with no suggestion of sheen but sometimes concentrically ringed. The two latter types predominated among the cultures studied.

Biochemical characteristics (at 30°C)

Catalase:—This enzyme is produced by all cultures.

Fermentation of glucose:—The end-products characteristic for the genus

Escherichia are formed. Carbon dioxide and hydrogen gases are formed in approximately equimolar proportions (*gas ratio 1:1*) besides significant quantities of ethanol and acetic, lactic and succinic acids (*methyl-red test positive*) with only traces of formic acid. Acetylmethylcarbinol and 2–3 butylene glycol have not been found (*Voges-Proskauer test negative*).

Citrate utilization:—Salts of citric acid are utilized as a sole source of carbon in an otherwise inorganic medium.

Hydrogen sulfide:—Not detected in proteose peptone ferric-citrate agar.

Indole:—May or may not be formed in tryptophane broth cultures.

Nitrates:—Reduced to nitrites.

Xylose, arabinose, rhamnose, glucose, fructose, mannose, galactose, lactose, maltose, trehalose and mannitol decomposed with acid or acid and gas production by all cultures. Melezitose, amygdalin and erythritol not attacked.

Sucrose, raffinose, cellobiose, α-methyl glucoside, adonitol, dulcitol, glycerol, inositol, sorbitol, starch, aesculin, salicin and sodium malonate attacked by some cultures and not by others.

Requirements for growth

Oxygen relationships:—Facultative.

Temperature requirements:—Growth occurs at 10°C and at 45° to 46°C. The optimum temperature for growth in the media used for study is between 30° and 37°C. Although some cultures show growth at 45° to 46°C gas is not produced in Eijkman test media when incubated at 43° to 46°C (temperature of the medium).

Salt tolerance:—Most cultures ferment glucose in the presence of sodium chloride in a concentration of 6.0 to 7.0. A few cultures tolerate 8.0 per cent sodium chloride.

pH range:—Cultures grow best in nearly neutral media although growth has been observed at pH 5.0 and pH 8.0.

Distinguishing characteristics

Cultures of *Escherichia intermedium* are differentiated from *Escherichia freundii* by the lack of ability of the former to produce hydrogen sulfide in proteose peptone ferric-citrate agar.

Two varieties of *Escherichia intermedium* have been observed. One was composed of 21 cultures which were unable to decompose starch or inositol but were motile. The other consisted of 6 cultures which decomposed starch and inositol with formation of acid and gas but were non-motile.

As already stressed it is not desirable to separate the H_2S negative *Escherichia intermedium* group into two varieties until a much larger number of such isolates have been available for study.

Sources of isolates

Cultures of *Escherichia intermedium* have been isolated from water, feces, soil, milk and olives.

The characteristics which separate *Escherichia intermedium* and *Escherichia freundii* from true *Escherichia* (citrate negative) and *Aerobacter* have already been given in table 2. Cultures of *Bacterium freundii* (Braak) received from Professor Kluyver include both sulfide-producing and non-sulfide producing types. The marked differences between the original cultures make it advisable to give specific names to the two types, so that the differences may be more easily noted.

The question of proper synonymy is difficult. Certainly "Intermediate" coli-like bacteria had been described under a variety of species of *Escherichia*, *Bacterium* or *Bacillus* for some time prior to the work of Koser (1923), Braak (1928), and Werkman and Gillen (1932).

Until descriptions included utilization of citrate as a sole source of carbon, the production of hydrogen sulfide in peptone, lead acetate agar offered the best clue as to whether the organisms might belong to the "Intermediate" section provided that other reactions were characteristic for coli-like bacteria. A study of the literature shows that it would be very difficult, if not impossible, to establish synonymy in this manner.

The most probable synonyms are those species of *Citrobacter* named by Werkman and Gillen (1932) including *Citrobacter album*, *Citrobacter anindolicum*, *Citrobacter decolorans*, *Citrobacter diversum*, *Citrobacter glycologenes* and *Citrobacter intermedium*. Had the trinomial *Bacterium coli citrovorum* (Minkewitsch, 1930) been used as a binomial as *Escherichia citrovorum* the "Intermediate" coli-like bacteria would have been given a very descriptive name.

It is not desirable at this time to separate the H_2S negative group into two varieties. A much larger number of strains should be available for study before stressing subdivision on statistical correlation.

E. Some ecological implications

Although it has been claimed that the "Intermediate" coliform bacteria (genus *Citrobacter*) are probably not normal inhabitants of the vertebrate intestine, it should be pointed out that such a conclusion is not warranted.

The data summarized in table 6 have been compiled from the literature for the purpose of ascertaining in part the relative incidence of the "Intermediate" section among the coliform bacteria isolated from different sources and also for pointing out the confusion which accompanies the presence of this group when isolated in large numbers from various sources. The results are based upon differentiation through the use of the methyl-red, Voges-Proskauer and Koser's citrate tests.

The data in table 6 indicate that the "Intermediate" section is widely distributed in nature; that it is encountered only in small numbers in the feces of man and animals; that the question of sanitary significance of this group is difficult to ascertain; that on the basis of the available data there is a somewhat higher incidence of this type in soils considered to be remotely polluted but the percentage incidence is low in most cases, showing little correlation with source and with relatively small percentage differences in incidence from various sources,

the conclusion that the "Intermediate" group is necessarily non-fecal in origin is unjustifiable.

Part of the somewhat higher percentage incidence among the non-fecal sources may be accounted for by the fact that with a predominance of *Aerobacter* forms there would be a larger number of such cultures incorrectly diagnosed as "Intermediate" because of the use of unreliable techniques for determining the V.-P. and particularly the methyl-red reactions, as for example the tendency to incubate at 37°C.

TABLE 6

Summary of incidence of "Intermediate" Coliform bacteria

SOURCE	NUMBER OF CULTURES	SECTIONS OF COLIFORM BACTERIA							
		Escherichia		Aerobacter		"Intermediate"		Unclassified	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Feces	5010	4308	86.0	273	5.4	354	7.1	75	1.5
Urine	453	235	71.7	172	38.0	46	10.3	0	0
Unpolluted soil	527	54	10.3	400	75.9	63	11.9	10	1.9
Remotely polluted soil	340	89	26.2	173	50.9	72	21.2	6	1.7
Recently polluted soil	1698	430	25.3	1078	63.5	146	8.6	44	2.6
Miscellaneous soils*	799	164	20.5	468	58.6	142	17.8	25	3.1
All soil isolations	3364	737	21.9	2119	63.0	423	12.6	85	2.5
Unpolluted water	4209	1441	34.2	1897	45.0	616	14.7	255	6.1
Polluted water	2452	1441	58.7	707	28.8	190	7.8	114	4.7
Miscellaneous waters*	10482	5880	56.1	2283	21.8	1694	16.2	625	5.9
All water insolations	17143	8762	51.1	4887	28.5	2500	14.6	994	5.8
Milk	2316	661	28.5	1147	49.5	470	20.3	38	1.7
Dairy products	195	88	45.1	81	41.5	26	13.3	0	0
Eggs	401	155	38.7	203	50.6	43	10.7	0	0
Hay and grain	723	114	15.8	532	73.6	64	8.8	13	1.8
Olives	156	0	0	110	70.5	46	29.5	0	0

* Not possible to differentiate type of water or soil from which cultures were isolated.

III. DISCUSSION

The "Intermediate" cultures herein reported were collected and studied over a ten-year period. During this time, no unusual characteristic irregularities or "variants" have been noted except those which were discounted as due to previous incorrect allocation. As already emphasized, use of inadequate methods of determining the V.-P. reaction and the use of 37°C rather than 30°C for incubation accounted for most of the "variants."

It is important to note, however, that in one instance true *Escherichia coli* cultures had been previously diagnosed as "Intermediate" because Simmon's citrate agar made with tap water had been used for differential purposes. It is known that agar contains growth-promoting factors. Koser's citrate medium carefully prepared to avoid chemical contamination should be used for determining whether coliform cultures can use the citrate radical as a *sole source* of carbon.

IV. SUMMARY AND CONCLUSIONS

The "Intermediate" group consistutes a distinct section of the coliform bacteria and may be differentiated from both *Escherichia* and *Aerobacter*.

The "Intermediate" section differs from true *Escherichia* in that the former (1) utilizes the citrate radical as a sole source of carbon in an otherwise inorganic medium, (2) produces H_2S in proteose peptone, ferric citrate agar, (3) decomposes cellobiose, (4) uses urea as a sole source of nitrogen but does not use uracil, (5) does not grow well at 45°–46°C (Eijkman test) and (6) is not resistant to boric acid.

The "Intermediate" section may be differentiated from true *Aerobacter* in that the former (1) does not produce acetylmethylcarbinol from glucose (V.-P.), (2) is methyl-red positive, (3) generally produces H_2S in proteose peptone, ferric citrate agar, (4) does not utilize uric acid, uracil, yeast nucleic acid, allantoin or hydantoin as sole sources of nitrogen, but does use urea, and (5) does not decompose sodium malonate.

The "Intermediate" coli-like bacteria are markedly group specific in their reaction to bacteriophages and to metabolites from staled agar, as shown by the work of Powers and Levine.

The "Intermediate" bacteria are most closely related to the organisms of the genus *Escherichia* from the standpoint of the physiological similarity in the metabolism of glucose. For this reason the cultures studied were allocated to the genus *Escherichia*, stressing, however, that until the question of natural habitat of the "Intermediate" section has been more conclusively proved it is desirable to segregate such cultures from true *Escherichia coli* which is conceded to predominate in the feces of vertebrates.

On the basis of statistical correlation, two species of "Intermediate" coli-like bacteria were recognized. The predominant species, *Escherichia freundii*, was represented by 196 cultures. The remaining 27 cultures were described as *Escherichia intermedium nov. comb.* A new description of *Escherichia intermedium* is also given.

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