COLIFORM INTERMEDIATES IN HUMAN FECES

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Although foundation for the recognition of coliform intermediates was laid by Brown (1921), he did not utilize his findings for their demonstration. Brown was interested in mediums containing citrated blood and attempted to determine the effect citrate might have on the growth of organisms likely to be encountered in blood culture work. Koser (1923, 1924a, b, c, d, 1926) applied to coliform bacteria (Breed and Norton, 1937) tests involving utilization of organic acids and their salts. He found that *Bacterium coli* from feces could not utilize citrate as a sole carbon source in contrast to *Bacterium aerogenes*. Koser found, particularly in soil and water, organisms classed as coli by the criteria then used but which utilized citrate-carbon and, in this respect, resembled *B. aerogenes*. In 1924 Koser applied the term "intermediate" to these forms.

Recognition of such intermediate forms should make possible a better theoretical understanding of the entire coliform group, than which few in bacteriology are more complex or confused. Any fundamental advance, such as the intermediate concept, should enable sanitary science to define more accurately such terms as "pollution" and "potability" and to answer numerous practical questions posed in this field in recent years.

Parr (1936a, b, 1937) defined intermediates as coliform organisms "which have one or more coli characters and one or more of those attributed to aerogenes, and some 'intermediates,' including the typical fecal form, produce hydrogen sulphide." This definition permits the inclusion as intermediates of organisms

which are Voges-Proskauer positive, a feature we feel essential and justified by our data.

The three characteristics, defining coli and aerogenes, most emphasized in recent papers are the methyl-red and Voges-Proskauer reactions and the utilization of citrate. To these it is felt indol production should be added. Formerly, following Houston, a water was considered polluted if it gave a "lactose +, indol +" reaction and the "Flaginac" mnemonic of English sanitarians, defining the colon bacillus, analyzes into "FL," fluorescence in neutral-red broth; "AG," acid and gas in lactose broth; "IN," indol from tryptophane; and "AC," acidity with coagulation in milk.

Parr and Caldwell (1933a, b) did not include the Voges-Proskauer reaction, under the impression that this reaction shows perfect correlation with the methyl-red reaction (Levine, 1916) and for the sake of uniformity the bored latrine studies by Caldwell and Parr (1937) utilize the same classification. It is now known that many coliform organisms do not exhibit perfect correlation, that is, they are not necessarily methyl-red positive when Voges-Proskauer negative and vice versa. Hence, in the present project four tests, i.e., indol production, methyl-red and Voges-Proskauer reactions, and citrate utilization were used as fundamental differentials in the classification of coliform organisms into coli, intermediates and aerogenes.

To fix and facilitate expression of results we devised the mnemonic "Imvic," (Parr, 1936b). Thus *Bacterium coli* (*Escherichia coli*) is + + − −, which means that it is indol positive (I), methyl-red positive (M), Voges-Proskauer negative (V), and citrate negative (C). *Bacterium aerogenes* (*Aerobacter aerogenes*) is − − + +, which means that it is indol negative, methyl-red negative, Voges-Proskauer positive and citrate positive. Table 1, a compilation of the tests used by some of the workers in this field in the order in which they list the tests used, is evidence for the need of some such standardization and justification for the selection of the members of the Imvic quartet of tests.

The 16 types, which combinations of the four characters determine, are listed in table 2. In this table the types encountered by recent investigators are indicated.

TABLE 1

Summary of differential tests used in the study of coliform intermediates

Koser (1924)	M.R.	V.P.	Uric A.	Citrate
Kline (1930)	M.R.	V.P.	Uric A.	Citrate
Ruchhoft et al. (1931)	Indol	M.R.	V.P.	Citrate
Gray (1932)	M.R.	V.P.	Citrate	
Skinner and Brudnoy (1932)	Cello.	Citrate	Indol	Sucrose
	V.P.	M.R.		
Parr and Caldwell (1933)	M.R.	Indol	Citrate	
Bardsley (1934)	M.R.	V.P.	Indol	Uric A.
	Citrate			
Bigger (1934)	Indol	M.R.	V.P.	Citrate
Tittsler and Sandholzer (1935)	Cello.	Citrate	H_2S	A-M-D-G
Wilson et al. (1935)	M.R.	V.P.	Citrate	Indol
	Eijkman	Gelatin		
Raghavachari and Iyer (1935)	M.R.	V.P.	Indol	Citrate
Kline (1935)	M.R.	V.P.	Citrate	Indol
	Eijkman			
Griffiths and Fuller (1936)	V.P.	M.R.	Citrate	Uric A.
	Indol			
Parr (1936)	Indol	M.R.	V.P.	Citrate
Bartram and Black (1937)	M.R.	V.P.	Citrate	Indol
Hook and Hitchener (1937)	M.R.	V.P.	Indol	Citrate
	Cello.			
Carpenter and Fulton (1937)	Citrate	M.R.	V.P.	

Times the test appears in this tabulation:

Indol production	12
Methyl-red reaction	16
Voges-Proskauer reaction	15
Citrate utilization	17
Uric acid utilization	4
Cellobiose fermentation	3
Eijkman test	2
Hydrogen sulphide production	1
Gelatin liquefaction	1
Sucrose fermentation	1
Alpha-methyl-d-glucoside fermentation	1

This report is primarily concerned with coliform intermediates derived from three types of fecal material, i.e., specimens of fresh feces and certain of these specimens stored at ice box and at body temperatures. Throughout, isolation of cultures has been by direct plating. True, for each fresh specimen enrichment tubes

TABLE 2

Coliform types and their occurrence

	1	2	3	4	5	6	7	8	9
Coli									
++−−	*	+	+	+	+	+	+	+	+
−+−−			+	+	+	+	+	+	+
+−−−									
Intermediates									
+++−					+				+
++−+			+	+	+	+	+	+	+
−+−+		+	+	+	+	+	+	+	+
+−+−									
−−−−								+	
+−−+			+	+				+	
−++−			+				+		+
++++				+			+		+
+−++		+	+	+		+		+	+
−+++			+	+			+		+
Aerogenes									
−−−+			+	+			+		
−−+−			+			+	+	+	+
−−++		+	+	+	+	+	+	+	+

The formulae express in order the indol production, methyl-red reaction, Voges-Proskauer reaction and citrate utilization, i.e., the "Imvic reaction."

* 1. Koser reported five types, but did not utilize indol. Types cannot be exactly placed.

2. Minkewitsch.

3. Kline.

4. Ruchhoft et al. Recognized but four types, ++−−, −+−−, −+−+, and −−++ as fecal. Others held to be extraneous to feces or mixtures.

5. Skinner and Brudnoy.

6. Bardsley.

7. Bigger.

8. Wilson et al.

9. Parr.

of lactose broth were prepared, but they were not utilized unless direct plating failed to reveal coliform organisms. That this may happen has been shown by Parr (in press) and by Carpenter and Fulton (1937) who record occasional fecal specimens which

yield no coliform bacteria. It is felt that direct plating is the most accurate method for determining the actual flora present. The various coliform bacteria have different metabolic demands and responses. It is improbable that any enrichment method yields an entirely accurate picture of the material enriched.

The colonies chosen for study have been purified by serial inoculation into plain broth and plating on Endo's agar. Ruchhoft and co-workers (1931) rendered a valuable service in emphasizing the confusion which mixed cultures introduce into the coliform field. Due cognizance has been taken of this point.

TABLE 3

Strains repeatedly replated to check purity

TYPE	STRAINS TESTED	CHANGES
−+++	3	0
++−+	5	0
++++	6	0
+−++	6	2*
−−+−	2	0
++−−	1	0
−+−+	2	0

* At the close of the experiment the two strains of +−++ which had changed were recovered as −−++. When mixtures are plated one usually recovers the component strains. For these none was encountered.

Strains isolated which did not correspond with the types sanctioned by the Ruchhoft report were repurified and retested. In addition, as a check on the Ruchhoft point of view, which is that only four fecal coliform types exist, i.e., −+−−, ++−−, −−++, and −+−+, a number of strains were further subjected to detailed purification involving 28 serial transplants on various mediums including seven platings and pickings. In only two instances did the reactions change and in these cases we are not convinced that the cultures in question were mixtures. See table 3.

As Parr and Caldwell (1933b) and d'Herelle and Rakieten (1934) have suggested, the possibility of biochemical variation, in freshly isolated strains not yet acclimatized to laboratory me-

diums, cannot be overlooked. Among the coliform bacteria characters are from time to time lost or those in abeyance are regained. It is in part this property of the group that gives rise to the multiplicity of forms responsible for confusing results in the attempt to apply a rigid and detailed botanical type of taxonomy and makes it desirable to emphasize the "lumper" point of view rather than the "splitter" type of classification (Skinner and Brudnoy, 1932). The occasional appearance of a variant reaction may, in our opinion, be a tribute to an investigator's close touch with his strains rather than, a priori, evidence of his carelessness. Wilson and co-workers (1935) stated that most cultures positive to both the methyl-red and Voges-Proskauer reactions are mixtures. Our results (table 3 and table 4) do not confirm this view.

Purified cultures were tested according to the Imvic complex and in addition for their dissimilation of glucose, lactose, sucrose, dulcitol, inositol, salicin, cellobiose and alpha-methyl-d-glycoside, for the production of hydrogen sulphide, liquefaction of gelatin and action on milk. Gram and capsule stains and motility tests were made as indicated. In addition, a considerable number of strains were examined for their growth in boric-acid lactose broth, in sodium malonate broth, and in Jordan and Harmon's tartrate agar; for their reduction of methylene blue and for their fermentation of propylene glycol, adonitol, inulin, raffinose, mannitol, arabinose, rhamnose, maltose and xylose. Endo's agar was used for plating, Simmon's citrated agar for determination of citrate utilization and fermentation broths were prepared from Difco phenol-red broth base to which the requisite carbohydrate, glucoside or alcohol was added prior to autoclave sterilization. Levine's iron citrate medium was used for hydrogen sulphide determinations. The data on coliform intermediates from human feces are presented in table 4.

Tribute to the heterogeneity of the coliform intermediates has been paid by Werkman and Gillen (1932), Tittsler and Sandholzer (1935), Parr (1936a) and by Carpenter and Fulton (1937). In each case the reference is to intermediates of the $-+-+$ type which our data clearly show is the most important type.

In our collection of intermediates of this type we have 29 varieties. Of these, 21 are hydrogen-sulphide positive, four liquefy gelatin, one fails to ferment cellobiose, 14 fail to ferment alpha-methyl-d-glucoside and, when analyzed by their fermentations of sucrose and dulcitol into MacConkey types, six are "acidi-lactici," six are "communis," eight are "communior," and nine

TABLE 4

Fecal coliform intermediates

		FRESH FECES	ICE BOX STORAGE	37°C. STORAGE	TOTAL
Specimens		235	68	38	
Platings		235	351	183	
Strains studied		1987	1690	905	4582
Intermediates		153	473	139	765
Per cent		7.7	27.9	15.3	16.6
Types	+++−	1	0	0	1
	++−+	7	81	23	111
	−+−+	110	135	59	304
	−++−	0	6	0	6
	++++	7	122	9	138
	+−++	28	50	39	117
	−+++	0	79	9	88
Totals		153	473	139	765

Order of importance:

(1)	−+−+	−+−+	−+−+	−+−+
(2)	+−++	++++	+−++	++++
(3)	++−+ ++++	++−+	++−+	+−++
(4)		−+++	++++ −+++	++−+
(5)		+−++		−+++

are "aerogenes." This collection of $-+-+$ types includes strains from other than fecal sources, such as "infected pumps," milk, marine food, eggs, soil, water and animal pathology.

By contrast the intermediate types $-+++$, $+-++$, and $++++$ are quite homogeneous whereas the type $++-+$ though less heterogeneous than $-+-+$ is nevertheless much more so than $-+++$, $+-++$, or $++++$. None of these

intermediate types belongs to but one MacConkey group. All $-+++$, $+-++$ and $++++$ strains and all but one $+++-+$ strain we have encountered ferment sucrose. The split which gives rise to more than one MacConkey group is found in the dulcitol fermentation. All these types, then, are found as both "communior" and "aerogenes" and in addition we have encountered a "communis" type $++-+$. The four intermediate types in question ferment cellobiose and alpha-methyl-d-glucoside and do not liquefy gelatin or produce hydrogen sulphide.

TABLE 5

Tabulation of tests designed to reveal coliform relationships

TYPES	M.B.-REDUCTION			PROPYLENE GLYCOL			SODIUM MALONATE			ADONITOL			BORIC ACID		
	+	−	Total	+	−	Total	+	−	Total	+	−	Total	+	−	Total
++−−	10	1	11	2	78	80	0	125	125	15	64	79	204	0	204
−+−−	0	0	0	0	13	13	0	19	19	7	4	11	5	0	5
++−+	5	6	11	4	6	10	6	4	10	8	28	36	16	0	16
−+−+	34	28	62	46	14	60	11	63	74	2	164	166	40	0	40
++++	3	11	14	11	0	11	14	2	16	36	0	36	5	0	5
+−++	6	0	6	9	0	9	12	0	12	14	0	14	1	0	1
−+++	14	2	16	8	2	10	19	2	21	43	0	43	3	0	3
−−+−	4	2	6	14	0	14	0	14	14	0	16	16	0	0	0
−−++	5	0	5	46	11	57	64	2	66	5	16	21	48	15	63
−−−+	0	0	0	2	0	2	2	0	2	1	0	1	0	0	0
Totals....	81	50	131	142	124	266	128	231	359	131	292	423	322	15	337

(Exceptions in the $++-+$ type). All $-+++$, $+-++$, and $++++$ ferment inositol and adonitol whereas the $++-+$ does not ferment inositol and most strains fail to ferment adonitol. It is interesting to note that the heterogeneous $-+-+$ type exhibits more homogeneity with respect to adonitol dissimilation than to any other test of differential value we have encountered, all but two of 166 strains failing to ferment that alcohol. The $+-++$ type is called aerogenes by a few workers. Table 5 gives the data for adonitol fermentation for some of the strains studied and includes other fragments of data which may interest students of the intermediates.

An important question is that of classification of the coliform intermediates. On this point Koser did not commit himself. Minkewitsch (1930) would recognize five species of coliform organisms as follows:

Coli sub-group:

Type I. *B. coli-communis* Escherich. Including all of its varieties and even the lactose-defective races of paracoli which are connected with typical coli by the intermediate form *B. coli-mutabile.*

Type II. *B. coli-citrovorum* Koser. Includes only the −+−+ type of intermediate.

Type III. *B. coli-anaerogenes* Lembke. Acid produced, but no gas.

Aerogenes sub-group:

Type IV. *B. aerogenes* Escherich.

Type V. *B. cloacae* Jordan.

Minkewitsch's terminology requires, of course, a change in genus from *Bacillus* to *Bacterium* and we feel his intermediate species is too narrowly conceived. His concept of the inclusion of paracoli and mutating coli with typical coli seems an excellent idea. We question whether his restriction of the intermediates, the anaerogenous coli and the cloacae to a habitat in cold-blooded animals can be justified. Furthermore it is hard to justify the establishment of a coli-anaerogenes species when paracoli are included with coli. It is to be regretted that in the coliform field no application can be made of animal pathology tests and that immunological procedures have as yet found but little utilization.

Werkman and Gillen (1932) have sought to erect a genus *Citrobacter* for organisms of the coliform group producing trimethylene glycol. They regard citrate utilization, the most widely used test in dealing with intermediates, as opposed to allocation with the citrate-negative colon bacilli.

Tittsler and Sandholzer (1935) divide the coliform bacteria into the genera *Escherichia* and *Aerobacter* on the basis of the

Voges-Proskauer reaction. As they conceive them the intermediates would then be classified as *Escherichia*. Although we cannot agree with their definition of intermediates their solution of the problem of classification could be accepted, some intermediates going to *Escherichia* and some to *Aerobacter*. More fundamental, as we see it, are the objections to breaking down the genus *Bacterium*; to including citrate-positive organisms with the coli; and to the emphasis placed on the Voges-Proskauer reaction, which is based on a quantitative rather than a qualitative differentiation. Carpenter and Fulton (1937) favor the inclusion of coliform intermediates in existing genera. They hold that coli and aerogenes are distinctly different and they believe the utilization of citrate not fundamental enough to prevent classing intermediates in the same genus with coli. They suggest a classification based primarily on the Voges-Proskauer reaction, secondarily on citrate.

Our experience compels us to regard citrate utilization as a sounder basis for classification than the Voges-Proskauer reaction. Koser (1924c) made a careful study of the stability of the utilization of citrate test. He found that under a wide variety of conditions the ability to utilize citrate is not readily lost when present or acquired when not present. With this conclusion we are in substantial agreement.[1]

It is further felt that cloacal forms of aerogenes differ sufficiently from the typical aerogenes so that any consideration of separate genera should consider a split in that direction. The gelatin-liquefying, motile, unencapsulated, glycerol-negative, propylene-glycol-negative, acetyl-methyl-carbinol-negative (as regards fermentation), chinic-acid (Butcher, 1926) negative cloacae demand taxonomic contrast to the aerogenes type with these reactions reversed. Jordan (11th Edition, 1935) felt that the splitting of the genus *Bacterium* into *Escherichia* and *Aerobacter* was not warranted. To this point of view we also subscribe.

Levine (1918) emphasized the desirability for restricting spe-

[1] Rare exceptions which we have encountered are under study and will be the subject of a later report.

cies numbers through a rigid application of methods of correlation. Unless points emerge distinctly advantageous for dairy, food and soil science, for sanitation and for medicine by the erection of new genera and species their construction seems unwise. This point is particularly pertinent for bacteriology, in which field botanical and zoological methods of classification only go so far, beyond which the microbes are lifted from their natural habitat and subjected to a wide variety of tests, selection of which has to be made with the greatest of care in order to elicit data of real value and to avoid undue complexity and confusion.

Among bacteria life spans are so short that many generations pass before the observer in a few days and without the averaging, leveling influence of sexual conjugation. In a group like the coliform bacteria where contributions by animal pathology and immunological methods are largely lacking and where opportunity for bacteriophage activity is so great it seems desirable to define the organisms encountered in simple and inclusive terms. This we feel could be done with four species of the genus *Bacterium* which would include coli, intermediates, aerogenes and cloacae and their varieties.

The coliform intermediates cannot be restricted to non-human sources as Minkewitsch (1930) has suggested. In a study of the coliform flora of 446 fecal specimens Carpenter and Fulton (1937) found one in which the only organism present was an intermediate type; 14 specimens where only intermediates and aerogenes were present; 16 yielding only intermediates and coli; and 31 which contained intermediates, aerogenes and coli. Thus they found intermediates in 62 specimens of 466 studied (13.3 per cent). Parr (In press) working on the general problem of the entire coliform flora and with a wider definition of intermediates than Carpenter and Fulton used found that 21.6 per cent of 235 fecal specimens contained intermediates. Of the 100 persons submitting these 235 specimens, 31 had a flora at one time or another containing intermediates. Parr found one specimen containing only intermediates, five containing aerogenes and intermediates, 25 containing intermediates and coli, and 19 specimens contained all three sections of the coliform group.

Parr (In press) has observed that there is considerable variation in the coliform flora of the same individual from day to day. Occasionally no coliform organisms at all may be recovered. Following such a period of time, which may include several days, coliform organisms again appear. It is at such "flora crises" as these that one is apt to encounter intermediates in very large numbers. They seem to be a stage in the re-establishment or balancing of the coliform flora. At other times they are not present at all or only in small numbers. Parr feels there is evidence for believing that the human colon is not the ideal habitat for coliform intermediates. However, since they are present in from one in eight to one in four of all fecal specimens and at times in very large numbers they cannot be stripped of significance in sanitation as indicators of pollution with alvine discharges.

It is unfortunate that our knowledge of coliform intermediates does not simplify the problems of sanitary science. That such problems are somewhat clarified is true, for added evidence is given in favor of the point of view that all coliform organisms may be suspected as of human fecal origin. The occurrence of all types of coliform bacteria in the bowel is further evidence of their close and intergrading relationship. From the practical standpoint evidence points to the necessity for strict emphasis on the highest standards of personal and public hygiene as the only safeguard against the spread of enteric bacteria.

CONCLUSION

A study has been made of 765 strains of coliform intermediates derived from fresh and stored feces and of additional type strains from other sources. As a working basis for the study of coliform organisms four tests have been utilized, i.e., indol production, methyl-red reaction, Voges-Proskauer reaction, and citrate utilization. All organisms are theoretically to be considered as intermediates which occupy a position between coli and aerogenes possessing one or more characteristics of coli and one or more of aerogenes. In practice we have restricted the intermediates to those so classified by the four reactions used.

The complexity of the entire coliform group is recognized. It

is believed unwise to dignify the many differences between forms observed with taxonomic recognition. Classification for the entire group should be simple and might well be comprehended in four species of the genus *Bacterium* to include coli, intermediates, aerogenes and cloacae.

Whether coliform intermediates are essentially of fecal or non-fecal origin has not been determined. But it is certain that they do occur in stool specimens to such an extent that they must be considered for sanitary purposes as indicators of fecal pollution.

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