THE UTILISATION OF CITRATES AND THE FERMENTATION OF CELLOBIOSE BY STRAINS OF *BACTERIUM COLI* ISOLATED FROM HUMAN FAECES.

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It has been suggested by Koser (8, 9, 10, 11, 13) that strains of *Bacterium coli* which are of non-faecal origin are usually able to use citrates as a sole source of carbon and that, like *Bacterium aerogenes*, such strains from water should probably not be considered as indicating faecal pollution, since faecal strains are usually citrate negative. Others have found that the citrate test correlated better with the sanitary condition of the water than the methyl-red or the Voges-Proskauer tests. However, not all investigators are favourable to the citrate test.

Although not expressed in words, the data of Tonney and Noble (22) may be shown to indicate that the faecal strains of *B. coli* are not nearly all citrate negative. Likewise a paper just out of press by Gray (6) concluded, from data unfortunately not published in full, that the correlation described by Koser between habitat and citrate utilisation was not confirmed. On the other hand, in the same issue of the same journal, Burke-Gaffney (3) found only 2 per cent. of 278 strains isolated from faeces to be citrate positive. Both Burke-Gaffney's and Gray's work represented only a relatively few individuals. Pawan (18) also found few citrate-positive strains from human faeces. Hicks (7) found 7 out of 100 strains able to utilise citrate. Unfortunately the extensive work of Ryti (20) on 319 organisms isolated from human faeces representing only 79 individuals included no tests on citrate utilisation. If citrate-utilising strains are not to be considered as indicating faecal pollution it should first be shown that they are not often found in faeces. Likewise the new cellobiose test first suggested by Jones and Wise (5) to differentiate *B. coli* from *B. aerogenes* but later found by Koser (12) to correlate with the citrate test should be negative with most strains of *B. coli* isolated from faeces if the test is of advantage.

The following work was planned to answer three questions. What proportion of strains of *B. coli* from human faeces are able to utilise citrates as a sole source of energy? What proportion ferment cellobiose with production of acid and gas? Are the two tests correlated in organisms isolated from faeces?

Experimental.

Routine samples of faeces from a hospital and from students at the University were obtained, but always only one sample was used from one person. A loopful was emulsified in a tube of sterile tap water and streaked on American

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Public Health Association standard eosin methylene-blue agar. In order to pick the colonies at random, in 24 hours the colony farthest from the place where the streaking was started was picked and inoculated into lactose broth. In 24 hours this was plated again on eosin methylene-blue agar and a colony picked and transferred to agar slants. The slants were incubated and were then inoculated into peptone broth for indol test (paradimethylamidobenzaldehyde); Clark and Lubs' broth[1] for methyl-red and Voges-Proskauer tests; lactose, sucrose, and cellobiose broth for acid and gas; and Simmons (21) agar for citrate utilisation. If the cultures showed lactose positive, cellobiose negative, citrate negative, indol positive, Voges-Proskauer negative, methyl red positive, and negative or positive to sucrose, they were taken to be typical *B. coli* and probably pure. If not, they were inoculated back into lactose broth and reisolated by plating. This was repeated until all the cultural characteristics of each strain checked the previous test four times in succession. There is no doubt in our minds that the anomalous strains of many authors represent mixed cultures, an opinion forcibly put forward by Ruchthoft, Kallas, Chinn and Coulter (19). At any rate we believe the strains here reported are pure beyond cavil. The last time the citrate-positive, cellobiose-positive, or indol-negative strains were checked was, in most cases, more than a year after isolation.

It might be mentioned that we used the Simmons technique for demonstrating citrate utilisation instead of the ferric ammonium citrate agar used by Murray and Skinner (17) or the original Koser technique (8), only because of the simplicity and speed of the Simmons method. One of us during the past few years compared all three media with over 500 strains of organisms which utilise citrate and with more which did not, and found a perfect agreement by all three methods. Lewis (16) has found that the liquid citrate medium results duplicate those of the solid ferric ammonium citrate agar, and Burke-Gaffney found a perfect agreement between Koser's and Simmons' methods. However, the ferric ammonium citrate agar necessitates a prolonged incubation, and Koser's liquid media requires a very small inoculum, preferably from liquid cultures, and is sometimes difficult to read. Nevertheless, in order to anticipate objections, we used also Koser's media, with perfect agreement with Simmons' method. At least 48 hours should be the minimum time for either method

[1] The formula for Clark and Lubs (4) broth states specifically that Witte's peptone must be used. It has been found that the "Proteose-peptone" of the Digestive Ferments Co. will give identical results. To the certain knowledge of one of the authors, the ordinary peptone "Bacto-peptone" of the same company has been substituted in certain laboratories, and it should be emphasised that proper results cannot be expected when this is done. Perhaps the statements in Levine's much-quoted bulletin (15) and in "Standard Methods" that "Proteose-peptone, Difco, or Witte's peptone" should be used may have been responsible for this serious error. Many people refer to Bacto-peptone as Difco brand. The elimination of the comma between the words "Proteose-peptone" and "Difco" will make the meaning much clearer, since Difco "Proteose-peptone" is not the same as the Difco "Bacto-peptone." Very probably the use of the wrong brand of peptone with a different buffer index has been responsible for some of the failures to correlate the Voges-Proskauer and methyl-red tests.

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before a negative test is recorded. Ninety-six was used by us, although most positive cultures could have been read in 24 hours.

DISCUSSION.

From Table I it can be seen that while most (501) of the 585 strains were cellobiose and citrate negative, of the 57 cellobiose-positive strains, 20 were citrate negative, and of the 64 citrate-positive strains, 27 were cellobiose negative. Thus there is little correlation with positive strains between tests. About 14 per cent. were either citrate or cellobiose positive, about 10 per cent. cellobiose positive, and 10 per cent. citrate positive.

Unless false negative results are to be expected, it would seem that the use of citrate or cellobiose should not be of much use in sanitary water analysis, as positive citrate or cellobiose tests are even more frequent than negative

Table I. *Cultural characters of* 585 *strains of lactose-fermenting aerobes each isolated at random from faeces of different persons.*

Number obtained	Cellobiose	Citrate	Indol	Sucrose	Voges-Proskauer	Methyl red
280	–	–	+	–	–	+
212	–	–	+	+	–	+
1*	–	–	+	–	+	+
4	–	–	–	+	–	+
4	–	–	–	–	–	+
4	+	–	+	–	–	+
15	+	–	+	+	–	+
1*	+	–	+	+	+	+
28	+	+	–	+	–	+
6	+	+	+	+	–	+
2	+	+	–	–	–	+
1†	+	+	–	+	+	–
11	–	+	+	+	–	+
6	–	+	–	–	–	+
10	–	+	–	+	–	+

* Uric acid –
† Uric acid +, 0·17 %
} 0·51 %.

indol tests. Koser's data show that the methyl-red positive, citrate-positive strains are fairly abundant in soil, and that they may give false positive tests using standard methods and Voges-Proskauer methyl-red reactions, but this was not confirmed by Gray. Even if citrate-negative strains are rare except in faeces, in case one has to choose between two evils, the margin of safety would require that false positives were preferable.

Table II, taken from Levine (15) with additions of data recorded since 1921, shows that *B. coli* is found in faeces in far greater numbers than *B. aerogenes*. Some of the isolations were by direct plating or streaking and some following preliminary enrichment. The criterion of separation of *B. coli* from *B. aerogenes* was one or more of the available tests; gas ratio, uric acid utilisation, Voges-Proskauer reaction, or methyl-red test. The reason for failure to correlate the tests occasionally recorded is very often, in our opinion, due to faulty technique. Ruchthoft and associates have stressed the commonness of impure cultures. For this reason we have adopted the tedious method of multiple plating until

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characters checked on several successive incubations. Again sufficient time for the methyl-red and Voges-Proskauer reactions has not always been allowed, as suggested by Gray. The use of improper peptone, as previously stated, wrong incubation time or temperature, impure phosphate, and other factors cannot but influence the results. Although we do not question the existence of "non-correlating" strains, in the experience of one of us covering more than a thousand strains, we cannot but conclude that strains failing to check in Voges-Proskauer and methyl-red reactions are rare indeed, providing adequate attention is given to purity of the cultures and proper technique. This has also been the experience of other investigators.

Table II. *Incidence of* B. aerogenes *among colon bacilli in human faeces**.

Investigator	No. of strains studied	Percentage *B. aerogenes* Voges-Proskauer positive, methyl-red negative, high CO_2/O_2, or uric acid positive
MacConkey	241	1·7
Ferriera, Horta and Paredes	178	5·6
MacConkey	117	6·8
Archibald†	100	0·0
Clemesha	1207	4·6
Levine	25	0·0
Hulton	10	0·0
Rogers, Clark and Lubs	113	5·8
Darling	20	0·0
Wood	33	0·0
Stokes	141	16·3
Leiter (14)	143	0·0
Ryti (20)	314	1·6
Skinner and Brudnoy	585	0·5
Burke-Gaffney (3)	278	3·0
Brown and Skinner (2)‡	157	0·0
Brewster (1)	385	1·8
Hicks (7)	100	5·0
Total	4147	3·3 % (average)

* Partly compiled from Levine, those entries being omitted which contained data from animal strains or from which the cultures were isolated by special methods. The figures given by Levine have not been checked.

† Includes strains isolated from latrines, faeces, and faecal suspensions exposed to sunlight.

‡ These strains originally isolated on eosine methylene-blue agar plates have since been confirmed by methyl-red and Voges-Proskauer methods.

Since Werkman and Gillen (23) have recently created a new genus *Citrobacter*, to include the "intermediate" group of methyl-red positive, Voges-Proskauer negative, citrate-positive, *B. coli* strains, their work should require a careful consideration. They did indeed find a correlation, *i.e.* citrate utilisation and tri-methylamine-glycol production, but unfortunately the technique given for demonstration of the latter requires equipment hardly to be found in many bacteriological laboratories. If a high correlation can be shown to exist in a hundred or so strains, we are willing to admit that there may be reason for creating a new *species*, although we doubt the practical advantage under our present knowledge of habitat of these bacteria. Unless a correlation of characters is insisted on for recognition of species, the number of species in a genus

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is likely to be multiplied by two every time a new test is proposed. Werkman and Gillen studied 15 strains of which six were stated to be isolated from soil and five from faeces. Of the soil strains it would seem that two cultures of the six gave fairly typical *B. aerogenes* reactions (*i.e.* cultures 18 and 18 *a*, faintly positive Voges-Proskauer and intermediate to negative methyl-red tests). Since among botanists and zoologists, there are traditionally two schools of taxonomists, the "splitters" and the "lumpers," the former who tend to recognise new species on slight grounds and the latter who tend to recognise species only when it cannot be avoided, bacteriologists will do well to realise that they are free to accept either point of view. One may be a perfectly good biologist (and a taxonomist as well) without recognising every new species and genus now being created at such an alarming rate, accepting only those for whose creation he sees good reason. To the authors it seems that from a pragmatic as well as scientific viewpoint the lactose-fermenting Gram-negative rods, capable of growing aerobically and of forming acid and gas from lactose, are still best divided into the species *Bacterium aerogenes* (Escherich) Chester, and *Bacterium coli* (Escherich) L. and N., and possibly also *Bacterium pneumoniae* (Friedländer) L. and N. *B. coli* and *B. aerogenes* are separated on the basis of several rather well-correlated characters as well as on the basis of habitat. Possibly the only reason for recognition of the group known as Friedländer's bacillus is usage, convenience, and pathogenicity. More and more bacteriologists are using the common or trivial name of an organism rather than the Latin binomial so that it may be known what they are talking or writing about. This unfortunate but almost unavoidable tendency will probably continue until the "lumpers" again gain ascendancy as they are doing in botany, especially in the field of mycology, *e.g.* Thom in *Penicillium* and *Aspergillus*, and Waksman and Jensen in *Actinomyces*.

SUMMARY.

1. Of 585 strains of lactose-fermenting aerobes each isolated from faeces of a different person, 501 did not utilise citrate or ferment cellobiose; 20 were citrate negative and cellobiose positive; 27 were cellobiose negative and citrate positive; and 37 were positive to both tests.

2. Almost 10 per cent. of the strains isolated were indol negative.

3. One culture was methyl-red negative and Voges-Proskauer positive. Two were positive to both tests. The rest were methyl-red positive and Voges-Proskauer negative.

4. It is concluded that the results are not favourable to the use of the citrate or the cellobiose test in routine water analysis.

5. A discussion of the taxonomy of the lactose-fermenting aerobes is given. Only two species, *Bacterium coli* (Escherich) L. and N., and *Bacterium aerogenes* (Escherich) Chester, are recognised.

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