THE DISTRIBUTION AND SANITARY SIGNIFICANCE OF *B. COLI*, *B. LACTIS AEROGENES* AND INTERMEDIATE TYPES OF COLIFORM BACILLI IN WATER, SOIL, FAECES, AND ICE-CREAM.

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(With 4 Graphs in the Text.)

CONTENTS.

I. LITERATURE.

The significance of *B. coli*, *B. lactis aerogenes* and the intermediate types of coliform bacteria in the examination of water and food, and the most profitable routine tests to employ for their detection, are still controversial questions among sanitary bacteriologists. More detailed information is required regarding the ecology of the coliform group in order that type may be correlated with source, and it has become important that only those tests giving the closest possible correlation with habitat should be used in routine examination. When the methyl red (MR) and the Voges-Proskauer (VP) tests were introduced, it was believed that by their use the lactose-fermenting bacilli could be divided into two groups which differed in their normal habitat. The true *B. coli* (MR_+VP_0) was thought to be of intestinal origin, while the *aerogenes* (MR_0VP_+) was said to be derived from soil. Recent workers have shown, however, that this distinction is not quite as definite as it appeared to be at first. The MR_+VP_0 organisms have been recovered from waters where the possibility of faecal contamination was not apparent, and the $MR_0\ VP_+$ type of bacillus is by no means confined to the soil, but is also present in the faeces of man and certain animals. In addition new tests have been introduced proving the existence of certain organisms, undoubtedly coliform, which cannot be assigned to either group but seem to occupy an intermediate position.

The investigation reported here has had for its object the examination of these problems. It is based on the study of 5181 strains of coliform bacteria,

https://doi.org/10.1017/S0022172400034380 Published online by Cambridge University Press

of which 4333 were derived from water, 152 from soil, 331 from faeces and 365 from ice-cream. All were Gram-negative bacilli producing acid and gas in lactose and clotting milk; they were classified by the use of the MR and VP reactions, Koser's citrate and uric acid media and the production of indol.

In a previous communication (Bardsley, 1926) a survey of the earlier literature was given in which the use of the methyl red and the Voges and Proskauer tests was fully discussed, but it has since been shown that cultivation in citrate medium, suggested by Brown (1921), gives a better correlation between the type of organisms and its source than the reactions formerly employed. Koser (1923, 1924, 1924 *a* and *b*, 1926) used the citrate test in the study of coliform organisms isolated from faeces, soil and water, and found that the ability to grow in the medium was a useful means of differentiating between MR_+ strains from soil which were mostly citrate utilisers (intermediate type), and MR_+ strains from faeces which generally failed in the test. In water analysis the percentage of MR_+ organisms was approximately the same in polluted and in unpolluted samples. The application of the citrate test, however, greatly reduced the proportion of faecal strains in the unpolluted supplies.

These observations were then applied to routine water analysis in the tropics, where, even after the use of the MR and VP tests, a marked discrepancy often existed between the results of the sanitary survey and the results of the bacteriological examination. Many waters which were used daily for drinking and domestic purposes without any ill-effect to health were shown to give very high *coli* counts if judged by Houston's standard or by the standard of the American Public Health Association.

Pawan (1925, 1926) confirmed Koser's findings with regard to the comparatively small number of citrate utilisers in faeces, and showed also that among 210 strains from polluted streams only 9·1 per cent. were citrate positive, while a series of 240 strains taken from unpolluted water included 81·3 per cent. of citrate utilisers.

Cunningham and Raghavachari (1924, 1926), working in India, studied the proportions of MR_+ and MR_0 organisms among lactose fermenters from various sources, and later Raghavachari (1926) added the citrate test in a series of soil and water examinations. The MR_0 type was predominant in soil, but the MR_+ type was predominant in water. In filtered supplies many of these low-ratio (MR_+) organisms were also citrate utilisers which failed in the indol test. The author concluded that it was not advisable to disregard the MR_+ $citrate_+$ type in assessing the purity of water.

Hicks (1927) in Shanghai found only 7·3 per cent. of 150 faecal strains to be citrate positive, but 80 per cent. of 50 soil strains were able to grow in this medium, and most of these failed to give indol. He suggested that the indol and citrate tests used together are of great sanitary value, since bacilli which are $citrate_+$ $indol_0$ might be regarded as non-faecal in origin. He did not consider, however, that the MR and VP tests alone were very helpful in deciding

whether contaminating organisms in water supplies were from excretal or non-excretal sources.

Having recognised the value of citrate as a useful distinguishing test among coliform bacilli, other selective media were from time to time introduced with the object of further classification.

Brown, Duncan and Henry (1924) suggested the use of tartrate peptone water. Jones and Wise (1926) correlated the fermentation of cellobiose, a β-glucoside, with the MR and VP reactions, and Koser (1926 *a* and *b*) found that most organisms which utilised citrate also gave acid and gas in cellobiose. Skinner and Brudnoy (1932) isolated nearly 11 per cent. of citrate utilisers among 585 strains from human faeces, but, unlike Koser, they were unable to establish reasonable correlation between the positive citrate and the positive cellobiose reaction. They considered that in water analysis the application of neither of these tests is justified. Werkman and Gillen (1932) studied the production of trimethylene glycol from glycerol by bacilli of the intermediate type (MR_+ VP_0 cit_+). They suggested that these organisms be given the generic name of *Citrobacter*, and they recognised seven distinct species within the genus. Levine and his colleagues (1932) added the glycerol reaction in a study of 401 strains of coliform bacilli from eggs. The strains were classified on the basis of citrate utilisation and the ability to produce trimethylene glycol. Koser and Saunders (1932) tested the growth of lactose fermenters from faeces and soil in α-methyl-*d*-glucoside, and concluded that this medium is of less value than citrate in distinguishing the intermediate organisms.

Burke-Gaffney (1932), of Dar-es-Salaam, East Africa, made an exhaustive study of 1923 coliform bacilli which were grouped as faecal, partly faecal and non-faecal types. He recovered MR_+ bacilli from many waters of known purity, but more than half of these were citrate utilisers in contradistinction to the MR_+ bacilli present in excreta. He believed that MR_+ cit_+ bacilli are typical of the soil flora, and that the occurrence of these organisms in water represents, at most, nothing more than remote pollution.

A similar investigation was carried out in Europe, where coliform organisms were never isolated from water or soil which, from sanitary survey, was believed to be free from pollution. The occurrence of *aerogenes* and the intermediate type (MR_+ cit_+) in the two series is of interest. Among 24 strains from remotely polluted soil in the tropics 67 per cent. were *aerogenes* and 29 per cent. were of the intermediate type, while among 24 strains from remotely polluted soil in Europe only 4 per cent. were *aerogenes* and 58 per cent. were of the intermediate type. On the other hand *aerogenes* was more common in faeces in Europe than in the tropics.

Gray (1932) contended that the distribution of coliform organisms in nature is "wide and intermingled," that *aerogenes* is not primarily a soil organism but is as typical of excretal flora as *B. coli*, although it may be present in smaller numbers. He quoted some of Cruikshank's unpublished results in which 78 out of 135 samples of human faeces yielded *aerogenes*, and was himself able to

isolate this organism from 37 out of 40 samples of faeces by first inoculating them into citrate medium and then plating out on MacConkey's agar. These results are supported by the earlier work of Hill and his colleagues (1929), who isolated more than 39 per cent. *aerogenes* (MR_0VP_+) among 200 strains of Gram-negative bacilli from genito-urinary infections, and by Burke-Gaffney (1932), who found that the citrate utilisers (*aerogenes* and the intermediate type) are predominant among lactose-fermenting bacilli in urine. On the other hand Tonney and Noble (1931) were able to demonstrate by the use of a direct plating method that the ratio of *B. coli* to *aerogenes* in faeces is greater than 100 : 1, while in soil and vegetation it is about 1 : 20. The same workers (1932) proved by sanitary survey that, as far as well waters are concerned, the occurrence of *aerogenes* in the absence of *B. coli* is not necessarily associated with faecal pollution.

In view of these results it becomes possible to apply one of two theories in the interpretation of the results of bacteriological examinations of food and water. Most workers in tropical countries where pollution is very heavy find it practicable to distinguish between the different types of coliform bacilli, and only those organisms which conform to the true *B. coli* group (MR_+ cit_0) are regarded as indicators of excretal contamination. In this manner the results of the bacteriological analysis are brought into line with the findings from sanitary survey and with the known quality of the water. It is stated that, if more stringent standards were applied in the tropics, many water supplies would be condemned which experience has shown to be safe for drinking.

In temperate climates the position is not so well defined. As early as 1912, when the classification of the colon group was much less complicated than it is to-day, Savage wrote: "Unless the use of elaborate classifications of *B. coli* shows a different distribution in Nature and a different significance, it cannot be said that these tests have any special value from the sanitary point of view." It has yet to be proved that classification on the basis of citrate metabolism fulfils these conditions. Some bacteriologists discount the citrate utilisers in assessing the purity of water. Others, who have found these organisms in faeces and in urine, insist upon the sanitary significance of the citrate-positive type, although it is doubtful whether their presence is indicative of recent pollution.

The accompanying tables give the results of the various investigators who have examined coliform bacilli from different sources. Table I classifies all the organisms on the basis of citrate utilisation alone, and Table II includes the MR and VP reactions as well as the citrate test, and the strains are classified into the *B. coli*, *aerogenes* and the intermediate groups.

II. WATER.

The study of coliform bacilli from water extends over a period of six years and deals with the results obtained from the examination of 2144 samples.

In the first section the series is considered as a whole, giving the results obtained from all the samples, and later, since all the waters were not used for

drinking purposes, the samples are grouped under four headings according to the nature and source of the supply.

Table I. *Results obtained by the use of the citrate test in the study of lactose-fermenting bacilli from different sources.*

Author	Date	Source	No. of samples	No. of strains tested	Percentage results: Not growing in citrate	Growing in citrate	Irregular strains
Koser	1924	Faeces	—	118	90·7	9·3	—
		Soil	70	72	2·8	97·2	—
	1924*a*	Unpolluted water	—	90	16·7	83·3	—
		Polluted water	—	107	64·5	35·5	—
Pawan	1925	Faeces	—	432	96·3	3·7	—
		Polluted water	—	210	90·9	9·1	—
		Unpolluted soil	—	214	10	90	—
		Unpolluted water	—	240	18·7	81·3	—
Koser	1926	Faeces	—	79	96·2	3·8	—
		Soil	—	162	11·1	82·1	6·8
	1926*c*	Unpolluted soil	41	104	23·1	76·9	—
		Polluted soil	11	33	63·6	36·4	—
Raghavachari	1926	Soil	54	518	5	95	—
		Filtered water	50	500	50·6	49·4	—
		Unfiltered water	108	1074	69·2	30·8	—
Hicks	1927	Faeces	—	150	92·7	7·3	—
		Soil	—	50	20	80	—
Burke-Gaffney	1932	TROPICAL RESULTS					
		Non-faecal (unpolluted water, unpolluted soil)	—	653	8	88	4
		Partly faecal (polluted water, polluted soil, cess pits, urine)	—	986	36	57	7
		Wholly faecal (faeces)	—	284	96	3	1
		EUROPEAN RESULTS					
		Non-faecal	—	—	—	—	—
		Partly faecal	—	432	70	26	4
		Faecal	—	145	87	12	1
Skinner and Brudnoy	1932	Faeces	—	585	89·1	10·9	—
Koser and Saunders	1932	Faeces	—	103	97	3	—
		Soil	—	53	0	100	—
Levine and colleagues	1932	Eggs	—	401	38·6	61·4	—

TECHNIQUE.

The *B. coli* examination was both a qualitative and a quantitative one. Preliminary inoculations in MacConkey's bile salt lactose medium were incubated at 37° C. for 48 hours, when a spread plate was made on MacConkey's agar from the highest dilution giving acid and gas, and three or more lactose-fermenting organisms were isolated. If more than one type of acid colony was present on the plates, representatives of each kind were tested. Strains which

were proved to be of typical morphology and staining reaction were submitted to the following confirmatory tests:

(1) *Lactose peptone water.* Incubation at 37° C. for the production of acid and gas.

(2) *Litmus milk.* Incubation at 37° C. Clotting usually took place within 24 or 48 hours. Cultures which failed to clot after 5 days were tested by boiling.

(3) *Peptone water.* Indol production was detected at first by the use of Böhme's reagent on a 5-day culture incubated at 37° C., but during the latter

Table II. *Previous studies on lactose-fermenting bacilli from different sources classified on the basis of the MR, VP and citrate tests.*

Author	Date	Source	No. of samples	No. of strains tested	Percentage results			
					B. coli	*B. lactis aero-genes*	Inter-mediate type	Irregu-lar strains
Koser	1924	Faeces	—	118	90·7	7·6	0·8	0·8
		Soil	70	72	2·8	50	22·2	25
	1926	Faeces	—	79	96·2	3·8	—	—
		Soil	—	162	11·1	64·2	17·9	6·8
	1926c	Unpolluted soil	41	104	23·1	67·3	7·7	1·9
		Polluted soil	11	33	63·6	33·3	3·0	—
Raghavachari	1926	Soil	54	518	5	93·4	1·5	—
		Filtered water	50	500	50·6	38·4	11·0	—
		Unfiltered water	108	1074	69·2	30·1	0·7	—
Burke-Gaffney	1932	TROPICAL RESULTS						
		Non-faecal (unpolluted water, unpolluted soil)	—	653	8	76	12	4
		Partly faecal (polluted water, polluted soil, cess pits, urine)	—	986	36	45	12	7
		Wholly faecal (faeces)	—	284	96	1	2	1
		EUROPEAN RESULTS						
		Non-faecal	—	—	—	—	—	—
		Partly faecal	—	432	70	6	20	4
		Faecal	—	145	87	9	3	1
Skinner and Brudnoy	1932	Faeces	—	585	88·72	0·17	10·77	0·34
Koser and Saunders	1932	Faeces	—	103	97	—	3	—
		Soil	—	53	—	50·9	47·2	1·9
Levine and colleagues	1932	Eggs	—	401	38·6	30·7	10·8	19·9*

* Classified as *Aerobacter cloacae.*

half of the investigation the Böhme test was discarded in favour of the oxalic acid test described by Holman and Gonzales (1923).

(4) *Glucose gelatine.* Stabs were made in glucose gelatine and incubated at 20° C. for 5 days.

(5) *Dextrose phosphate* (Clark and Lubs, 1915). Cultures were incubated at 30° C. for 5 days for the methyl red and Voges-Proskauer reactions.

(6) *Koser's uric acid test.* This medium was prepared according to Koser's formula (1918). All the tubes were specially cleaned in sodium hypochlorite or

in a mixture of dichromate and H_2SO_4 and then washed in tap water and in distilled water. Each batch of medium was carefully tested with stock cultures of coliform bacilli before use.

(7) *Koser's citrate test.* This medium was also prepared according to Koser's directions (1923) except that 2·7 g. of sodium citrate were added instead of citric acid and NaOH. The tubes used in this test were also specially prepared and washed, and each batch of medium was carefully tested.

Cultures in uric acid and in citrate were incubated at 30° C. for 5 days.

If none of the strains isolated from the first plating were true *B. coli* (MR_+ VP_0 $indol_+$ $uric_0$ cit_0), the remaining MacConkey tubes giving a positive presumptive result were plated out and further cultures were tested.

Results.

Reactions given by the strains taken as a whole without any reference to source.

Of the 2144 samples of water examined 1102 gave a positive result in MacConkey's bile salt lactose medium, and 4333 strains of coliform bacilli were isolated.

MR and VP tests.

The 4333 strains included 24 which gave anomalous reactions in the MR and VP tests; 22 gave a double positive and two a double negative result, and these reactions were maintained even after repeated replating and retesting. Many workers have isolated organisms giving MR_+ VP_+ or MR_0 VP_0 results, Johnson (1916), Burton and Rettger (1917), Koser (1918), Chen and Rettger (1920), Bardsley (1926) and Burke-Gaffney (1932). In the present study the number of irregular types was very low, being less than 0·6 per cent. of the total. These 24 strains were discounted along with 12 gelatine liquefiers, leaving 4297 organisms of which 3694 were MR_+VP_0 and 603 MR_0VP_+. The correlation of these results with the indol, uric acid and citrate tests and the classification of the strains into the *B. coli*, *aerogenes* and intermediate groups is given in Table III.

MR and the Koser tests.

The inverse correlation between the methyl red and the uric acid reactions was very well defined. Of the 603 MR_0 organisms 92·9 per cent. were able to grow in uric acid, and of the 3694 MR_+ organisms 98·1 per cent. failed to grow. In the case of the citrate test the correlation was much more clearly defined among the MR_0 strains, where 98·2 per cent. were citrate positive, than among the MR_+ strains, where 82 per cent. were negative and 18 per cent. were positive.

MR, Koser and indol tests.

Of the 3027 MR_+ VP_0 cultures which were negative in both uric acid and citrate 2947, 97·3 per cent., were positive in the indol test and were classified as *B. coli*. 597 MR_+VP_0 strains did not grow in uric acid but grew in citrate, and

of these 581, 97·3 per cent., were negative in the indol test. In view of this result the intermediate type was considered throughout as a MR_+VP_0 organism growing in citrate but not in uric acid and giving no indol in peptone water, and this type of organism formed 87·5 per cent. of the total number of citrate utilisers among the MR_+ cultures. A similar inverse correlation between the citrate and indol tests has been reported by Koser (1924), who found that 16 out of 25 MR_+ cit_+ strains from soil were indol negative, and Pawan (1926) discovered a close agreement between growth in citrate and failure to produce indol. Raghavachari (1926) reported complete inverse correlation between these tests among 518 strains from soil, eight of which were MR_+ cit_+, and 1574 strains from water, of which 63 were MR_+ cit_+. Hicks (1927) found that 68 per cent. of 50 MR_+ VP_0 strains from soil failed to give indol but grew in citrate.

Table III. *The correlation of the MR and VP reactions with the indol, uric acid and citrate tests.*

	Indol	Uric acid	Citrate	Type	Number
MR_+ VP_0 = 3694	−	−	−	Irregular	80
	+	−	−	*B. coli*	2947
	+	+	−	—	0
	+	+	+	Irregular	32
	−	+	+	Irregular	35
	−	−	+	Intermediate type	581
	+	−	+	Irregular	16
	−	+	−	Irregular	3
MR_0 VP_+ = 603	−	−	−	Irregular	7
	+	−	−	—	0
	+	+	−	—	0
	+	+	+	*B. lactis aerogenes*	78
	−	+	+	*B. lactis aerogenes*	478
	−	−	+	Irregular	36
	+	−	+	—	0
	−	+	−	Irregular	4

The indol test did not correlate well with the uric acid and citrate results among the MR_0 strains because a large proportion, 14 per cent., of the *aerogenes* type were indol producers.

Among the 4297 organisms isolated which gave normal MR and VP results and could be classified as coliform bacilli on the basis of the lactose, milk and glucose-gelatine tests, there were 2947 *B. coli* (MR_+ VP_0 $uric_0$ cit_0 $indol_+$), 581 intermediate type (MR_+ VP_0 $uric_0$ cit_+ $indol_0$), and 556 *aerogenes* (MR_0 VP_+ $uric_+$ cit_+ $indol_+$ or $indol_0$). The remaining 213 strains gave irregular results and could not be included in any of these three groups. Ruchhoft and his co-workers (1931) believe that many so-called irregular strains are really mixed cultures. In the present study organisms of this kind were always retested and their anomalous reactions were confirmed before they were finally added to the collection of lactose-fermenting bacilli reported here.

Delayed lactose fermentation and weak clotting power.

The majority of organisms were able to produce acid and gas in lactose and a clot in milk after 24 hours' or, at the most, after 48 hours' incubation at

37° C., but there was a certain proportion of strains which showed delayed lactose fermentation and weak clotting power. In cases where gas did not make its appearance in lactose within 48 hours the cultures were returned to the incubator for a further period of 6 or 8 days. Generally speaking gas was apparent after the fifth day, although often it scarcely filled 10 per cent. of the Durham tube. If no gas developed after 10 days' incubation the organisms were discarded.

The litmus milk cultures which showed acid but no clot were also incubated 10 days at 37° C.; then, if they still had not clotted, they were boiled for a few minutes in a water bath. All cultures of this type produced a clot on boiling.

Table IV. *Showing the percentage of strains giving delayed lactose fermentation and weak clotting power in milk.*

Strain	Total no. isolated	Percentage of strains giving: Acid and gas in lactose and clot in milk within 48 hours	Delayed lactose fermentation and weak clotting power	Delayed lactose fermentation	Weak clotting power
B. coli	2947	97·34	0·17	0·27	2·2
B. lactis aerogenes (indol±)	556	71·22	15·29	6·12	7·37
Intermediate type	581	47·33	35·80	11·02	5·85
Irregular strains	213	65·25	17·38	8·45	8·92
Total	4297	85·61	7·79	2·89	3·72

The incidence of these strains is given in Table IV. More than 97 per cent. of the 2947 *B. coli* and more than 70 per cent. of the 556 *aerogenes* were able to produce gas in lactose and a clot in milk after 48 hours' incubation at 37° C. Among the 581 organisms of the intermediate type, however, there were only 47·33 per cent. which were able to complete both these reactions within 48 hours. It was noticed also that the majority of the intermediate strains differed from true *B. coli* in morphology, being slightly shorter and thicker, but they were quite distinctly bacilliform and of definite staining reaction.

Bronfenbrenner and Davis (1918) studied late lactose fermenters from various foods and found that the production of gas within 24 hours could be induced by continual transference of the culture in lactose peptone water, or by increasing the concentration of lactose to more than 1 per cent. In the present study the medium contained only 0·5 per cent. lactose, and no experiments were carried out to discover whether an increase in the proportion of the sugar would induce more vigorous gas formation among the intermediate types.

Jones and his colleagues (1932) isolated a number of slow lactose fermenters from the faeces of cows suffering from infectious diarrhoea. Thirty-seven of the strains were classified on the basis of certain sugar fermentations together with the MR, VP, indol, gelatine and H_2S tests, and all gave reactions typical of *B. coli* (MR_+ VP_0 $indol_+$). The slow lactose fermenter found so frequently among the intermediate group in the present study seems not to have occurred.

https://doi.org/10.1017/S0022172400034380 Published online by Cambridge University Press

A comparison of the Böhme test and the oxalic acid test for indol.

566 coliform bacilli (413 *B. coli*, 55 *aerogenes*, 60 intermediate types and 38 irregular strains) were tested for indol by the Böhme method and also by the oxalic acid method described by Holman and Gonzales (1923), and it was found that the oxalic acid test was rather more delicate than the Böhme. Five organisms which showed only a trace of indol with Böhme gave a definite positive with oxalic acid, and seven organisms which were found negative by the Böhme test, even after the addition of potassium persulphate ($K_2S_2O_8$), were proved to be positive when the oxalic acid test was applied. These seven organisms were all MR_+ VP_0 $uric_0$ cit_0, thus conforming to the true *B. coli* type. They were also considered $indol_+$ on the basis of the oxalic acid test and were included among the 2947 *B. coli* strains. One organism gave a positive reaction with Böhme's reagent and a negative with oxalic acid, but, as it was an irregular type, it was impossible to discover which result was more in accord with the other characters of the strain.

Correlation of type with source.

The 2144 water samples can be divided into four main groups according to the source of supply.

Group I consists of 1622 drinking waters collected from town supplies. Most of them were upland surface waters gathered from peaty moorland districts in the north-west of England where there is little chance of contamination except by birds and wild animals, and many of them were treated by sand or mechanical filtration before delivery to the consumer. Only 750 (46·24 per cent.) of these samples gave a positive presumptive test in MacConkey, and 2572 strains of coliform bacilli were isolated. The remaining 872 samples (53·76 per cent.) yielded no *B. coli* in 100 c.c.

Group II consists of 399 samples, also drinking waters, which were derived from shallow wells, pumps or springs yielding only a small quantity of water used locally at farms and cottages too isolated to receive town supplies. None of these waters was artificially purified before use, and the proportion of positive samples was higher in this class of water. 286 samples (71·68 per cent.) gave acid and gas in MacConkey, and 1375 coliform bacilli were isolated.

Group III includes 90 samples of swimming-bath water. These samples varied very much in their *coli* content, depending on the kind of purifier used and also on the length of time which elapsed since the water was last treated. In 53 cases the samples were collected direct from the filter, and in these no coliform bacilli were found. 37 samples (41·11 per cent.), which were taken from the bath itself, were definitely polluted, and from these 231 lactose-fermenting organisms were isolated.

Group IV contains 33 "miscellaneous" samples, which, being derived from ponds, streams, fish docks and other polluted sources, could not be classified under any other heading. 29 (87·90 per cent.) of these samples were positive, and 119 strains were isolated.

Results considered according to strains.

A comparison of the proportions of the various types of coliform bacilli occurring in water from different sources is given in Table V.

Table V. *Classification of coliform organisms with regard to type and source.*

Source	Total no. of strains	*B. coli* $MR_+ VP_0 indol_+ uric_0 cit_0$ No.	%	*B. lact. aerogenes* $MR_0 VP_+ indol_\pm uric_+ cit_+$ No.	%	Intermediate type $MR_+ VP_0 indol_0 uric_0 cit_+$ No.	%	Irregular strains No.	%
Town supplies (upland surface)	2572	2008	78·07	194	7·54	279	10·85	91	3·54
Rural supplies (wells & springs)	1375	762	55·43	256	18·62	257	18·69	100	7·27
Swimming baths	231	95	41·12	81	35·06	35	15·17	20	8·66
Miscellaneous	119	82	68·92	25	21·01	10	8·41	2	1·68
Totals	4297	2947	68·57	556	12·94	581	13·52	213	4·96

The majority of the lactose-fermenting bacilli from upland surface waters were true *B. coli*, the percentages of the other types being comparatively small. In the samples collected from wells and springs, which are frequently polluted with soil washings, the relative number of *B. coli* was lower, and the percentage of citrate utilisers, believed by many workers to be typical of the soil flora, was about twice as great as in town supplies.

None of the swimming-bath waters were collected from open-air pools, so that direct contact between the soil and the water was impossible in every case, and yet they yielded a smaller percentage of *B. coli* and a higher percentage of *aerogenes* than any other group of samples. Out of 231 strains isolated, only 41 per cent. were *B. coli*, while 35 per cent. were *aerogenes* and 15 per cent. were of the intermediate type. It is obvious that the theory of the soil origin of *aerogenes* utterly fails to explain the high percentage of this organism in swimming-bath waters.

Gray (1932) carried out a number of experiments on the relative longevity of *B. coli* and *aerogenes* in water and found that the MR_0 type remained viable over a longer period than *B. coli*, and considered, therefore, that the presence of *aerogenes* in food or water meant nothing more serious than remote faecal pollution. The application of Gray's theory to the present results also fails to explain the facts. The pollution of swimming baths certainly cannot be regarded as "remote," on the contrary it is continually recurring, for as the filtered and disinfected water reaches the bath it is immediately recontaminated. Again, if the high proportion of *aerogenes* in water depended on the relative longevity of this organism compared with *B. coli*, one would have expected to find the largest number of MR_0 types in the upland surface waters, most of which had been stored for considerable periods in impounding reservoirs under conditions similar to those which Gray reproduced in his experiments.

Longevity is the result of resistance to unfavourable external forces, which, whether physical, chemical or biological, should be taken into account when assessing the viability of any particular organism. No experiments have as yet been carried out on these lines, but the results obtained here suggest that

aerogenes may offer a certain resistance to the action of chemical reagents, particularly to the various forms of chlorine used in bath waters, and may, therefore, be able to survive such treatment more easily than *B. coli*.

The strains from the "miscellaneous samples," chiefly pond and stream waters, also included a large number of *aerogenes*, but the percentage of this organism in swimming-bath waters was nearly twice as high.

Results considered according to samples.

It will be interesting to compare these figures with the results obtained when the percentage of samples giving the various types is considered in relation to the source of supply. These results are given in Table VI, the percentages being taken on the total number of samples giving a positive presumptive test in MacConkey's bile salt lactose medium.

Table VI. *Grouping of waters according to source and type of lactose-fermenting bacilli present.*

Source	No. of samples tested	No. giving a positive presumptive result	Samples which yielded: *B. coli* $MR_+ VP_0 indol_+ uric_0 cit_0$ No.	%	*B. lact. aerogenes* $MR_0 VP_+ indol_\pm uric_+ cit_+$ No.	%	Intermediate type $MR_+ VP_0 indol_0 uric_0 cit_+$ No.	%	Irregular strains No.	%
Town supplies (upland surface)	1622	750	647	86·26	58	7·73	87	11·60	40	5·33
Rural supplies (wells & springs)	399	286	229	80·05	57	19·93	57	19·93	35	12·24
Swimming baths	90	37	30	81·08	13	35·14	12	32·43	6	16·22
Miscellaneous	33	29	28	96·56	4	13·79	6	20·69	1	3·45
Totals	2144	1102	934	84·74	132	11·98	162	14·70	82	7·44

The most striking feature of Table VI is that in every series of samples no less than 80 per cent. of the waters giving a positive presumptive result yielded *B. coli*, and if the production of acid and gas in MacConkey's medium had been regarded throughout the investigation as sufficient evidence of the presence of this organism, only 15·26 per cent. of the positive samples would wrongly have been included among those contaminated in this way.

There were a number of waters in every series which gave more than one type of strain, and these are included more than once in the table. The more detailed results showing the various combinations of types are given in Table VII.

With the exception of *B. coli* the percentage of samples yielding only a single type of strain was low in every series. More than 80 per cent. of the positive samples among the town supplies contained no organism except *B. coli*, and 6 per cent. contained only the intermediate type, but the percentages giving *aerogenes* and the various combinations were uniformly low. The well waters also gave no figure other than *B. coli* which could be said to have any special significance.

In examining swimming-bath waters it rarely happened that *B. coli* was isolated among the first collection of strains. It was often necessary to plate

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out every positive MacConkey tube in the effort to isolate this bacillus. This is shown in Table VII, where, although the percentage of samples yielding only *B. coli* was lower than in any other series, the organism frequently occurred in combination with the other types, a fact which explains the high percentage of bath waters shown in Table VI to contain a bacillus of this kind. The flora of swimming baths was much more varied with regard to coliform organisms than the waters collected from other sources. This is the probable result of chemical treatment which appears to destroy *B. coli* more rapidly than *aerogenes*, so that the proportion of the MR_0 types increases.

Table VII. *The combinations of the various types of coliform organisms yielded by samples from different sources.*

	Source of supply							
	Town supplies (upland surface water)		Rural supplies (wells, pumps & springs)		Swimming baths		Miscell. samples	
No. of samples yielding								
No coliform bacilli	872		113		53		4	
Coliform bacilli	750		286		37		29	
	No.	%	No.	%	No.	%	No.	%
B. coli only	600	80·0	183	63·98	19	51·36	21	72·41
B. lact. aerog. only	20	2·67	9	3·15	3	8·11	0	—
Intermediate type only	47	6·27	18	6·29	1	2·7	1	3·45
B. coli and *B. lact. aerog.*	13	1·73	14	4·9	1	2·7	1	3·45
B. coli and intermed. type	12	1·6	6	2·1	2	5·41	2	6·90
B. lact. aerog. and intermed. type	15	2·0	12	4·2	1	2·7	0	—
B. coli, *B. lact. aerog.* and intermed. type	3	0·4	9	3·15	4	10·81	3	10·34
Irregular strains only	13	1·73	9	3·15	1	2·7	0	—
B. coli and irregular strains	11	1·47	7	2·45	0	—	1	3·45
B. lact. aerog. and irreg. strs.	4	0·53	4	1·4	1	2·7	0	—
Intermed. type and irreg. strs.	4	0·53	3	1·05	0	—	0	—
B. coli, *B. lact. aerog.* and irreg. strs.	2	0·27	3	1·05	0	—	0	—
B. coli, intermed. type and irreg. strs.	5	0·67	3	1·05	1	2·7	0	—
B. lact. aerog., intermed. type and irreg. strs.	0	—	2	0·7	0	—	0	—
B. coli, *B. lact. aerog.*, intermed. type and irreg. strs.	1	0·13	4	1·4	3	8·11	0	—

The polluted samples in the miscellaneous group included a high percentage which yielded only *B. coli*, and there was also a number which contained all three types of coliform bacilli.

Seasonal incidence.

It was considered that, as the work extended over such a long period, it might be possible to trace a seasonal variation in the occurrence of lactose fermenters in water, or even to detect a prevalence of certain types at different seasons of the year.

For the purpose of this study each group of drinking waters was considered separately on the basis of samples and of strains. There were too few swimming-bath and "miscellaneous" waters to make a special consideration worth while.

Results considered according to samples.

The results of all the samples received during all the Januarys, all the Februarys, and so on throughout the six years, were collected together and the monthly aggregate percentages on the monthly totals were plotted. Graphs I and II were thus obtained.

It will be noticed that the *B. coli* curves followed the graphs given by the percentage of positive samples very closely indeed, and there were more samples positive in summer than in winter. Also there was a decided tendency for the percentage of samples yielding *B. coli* to remain constant or to decrease in the winter and spring months, *i.e.* from about October to March, while at the same time the percentage of samples yielding *aerogenes* and the intermediate type showed an increase. In the late summer or autumn the reverse happened. The *B. coli* percentages increased and *aerogenes* and the intermediate type decreased or remained fairly constant.

Results considered with regard to strains.

The percentage numbers of *B. coli*, *aerogenes* and the intermediate type were calculated on the aggregate monthly totals and plotted in a similar manner.

Graphs III and IV give the seasonal incidence of the strains isolated from good drinking waters and of the strains found in doubtful waters. They show a definite decrease in the number of *B. coli* from January to April accompanied by a rise in the *aerogenes* and the intermediate type. In town supplies and in well waters the graph of the intermediate type was practically the inverse of that of the *B. coli*. In well water (Graph IV) the *aerogenes* was more numerous, and the maxima of this graph coincided very markedly with the minima of the *B. coli* curve. This is not wholly due to the technique, since two other variable quantities were present, namely the intermediate type and irregular strains.

It would appear from these results that there is some slight evidence of a seasonal variation among the lactose-fermenting bacilli in water, for, although *aerogenes* and the intermediate type never become dominant, yet they do show a relative increase in the late winter and spring months, and at the same time *B. coli* seems to die out. Conversely, in the summer and early autumn *B. coli* shows a definite increase and the other organisms become relatively less numerous. These findings are not in agreement with the results of Tonney and Noble (1931 *a*), who, in their work on infected stumps, found that under winter conditions *B. coli* and *aerogenes*, both from faecal material and from cultures, underwent rapid decline without showing any significant change in the relative numbers.

Summary of water results.

This section of the work deals with the routine bacteriological examination of 2144 samples of water, of which 1102 contained lactose-fermenting bacilli, 4333 strains being isolated. Twelve strains which liquefied gelatine and 24

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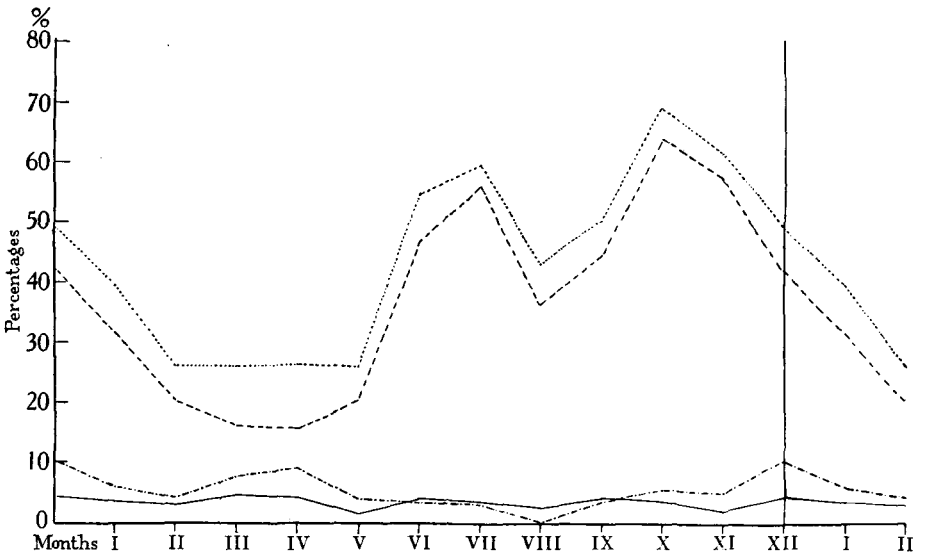
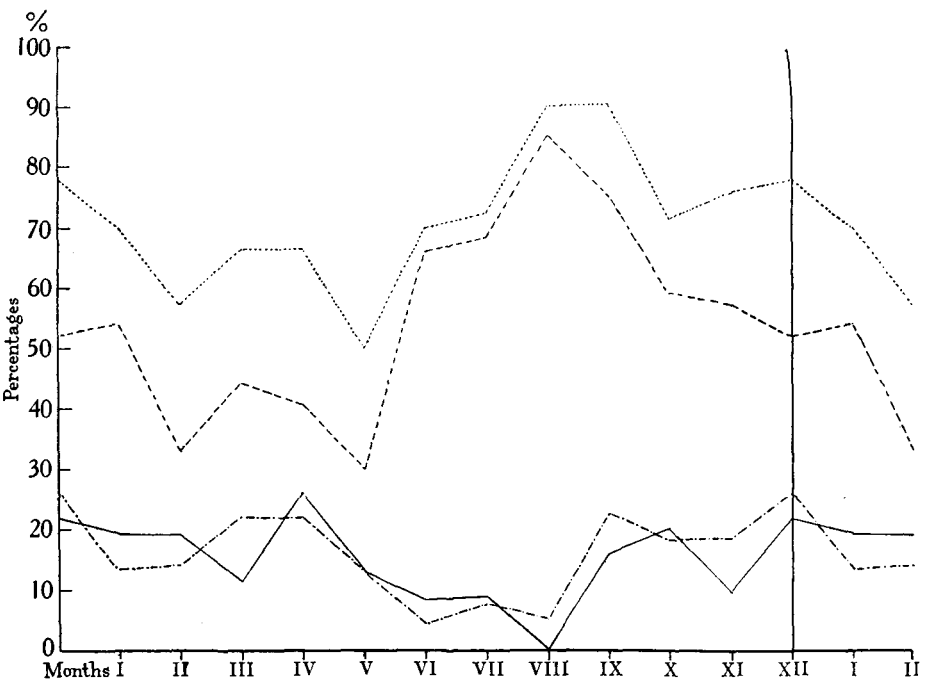


Graph I. Seasonal incidence. Town water supplies. Percentage of samples yielding coliform organisms.



Graph II. Seasonal incidence. Rural water supplies. Percentage of samples yielding coliform organisms.

..... Percentage of samples giving the presumptive *coli* test.
– – – – – Percentage of samples giving *B. coli* (MR_+ VP_0 $indol_+$ cit_0).
——— Percentage of samples giving *B. lactis aerogenes* (MR_0 VP_+ $indol_\pm$ cit_+).
–·–·–·– Percentage of samples giving intermediate type (MR_+ VP_0 $indol_0$ cit_+).

I–XII denote months Jan.–Dec.

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which gave anomalous MR and VP reactions were excluded from the coliform group; the remaining 4297 cultures were classified on the basis of the MR, VP, indol, citrate and uric acid tests.

A very close agreement was observed between the MR_+ organisms and the negative uric acid result. Out of 3694 MR_+VP_0 strains only 1·9 per cent. were able to grow in uric acid. The citrate test did not show such close correlation, 18 per cent. of the MR_+ strains being also citrate utilisers.

Indol production generally corresponded with the inability to grow both in uric acid and in citrate among the MR_+ strains, but a certain number, 14 per cent., of the $uric_+$ cit_+ cultures among the MR_0 strains were indol-positive.

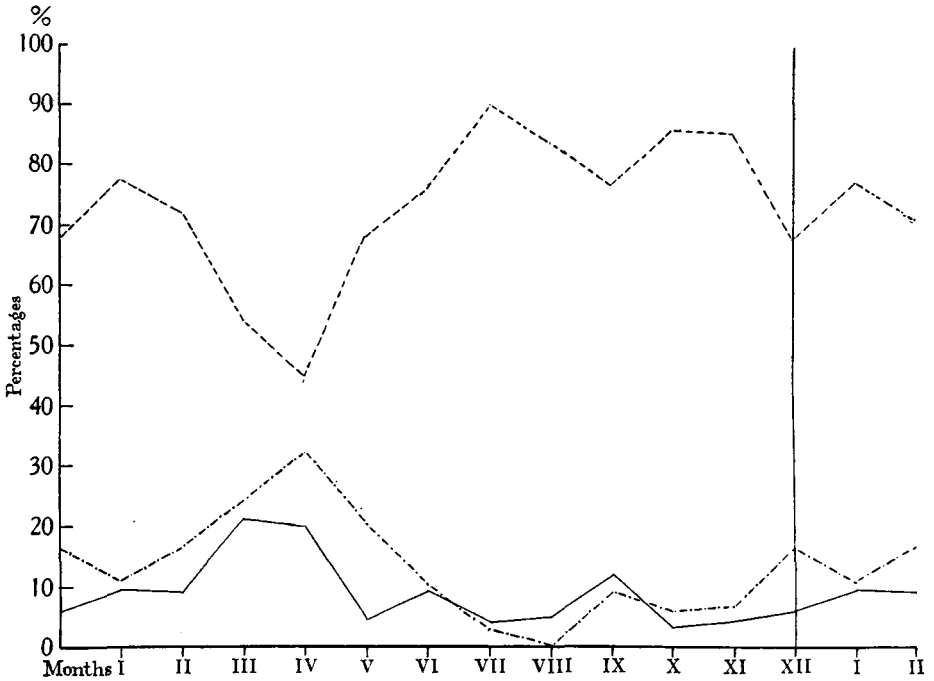
There were 597 MR_+VP_0 bacilli which developed in citrate but failed to grow in uric acid, and of these 581 (97·3 per cent.) produced no indol. Thus the intermediate type was considered throughout the investigation as a low-ratio (MR_+) organism growing in citrate, but not in uric acid, and giving no indol in peptone water. The 4297 strains isolated included 2947 *B. coli*, 556 *aerogenes* and 581 intermediate type; 213 cultures which gave irregular results and could not be classified were regarded for convenience as anomalous strains.

Weak clotting ability and delayed gas production in lactose was observed in many strains, particularly among the intermediate type, where only 47·33 per cent. of the 581 cultures were able to complete the lactose and milk reactions within 48 hours. It was also noticed that the intermediate type was usually shorter and thicker than *B. coli*, although it was quite definitely bacilliform and of definite staining reaction.

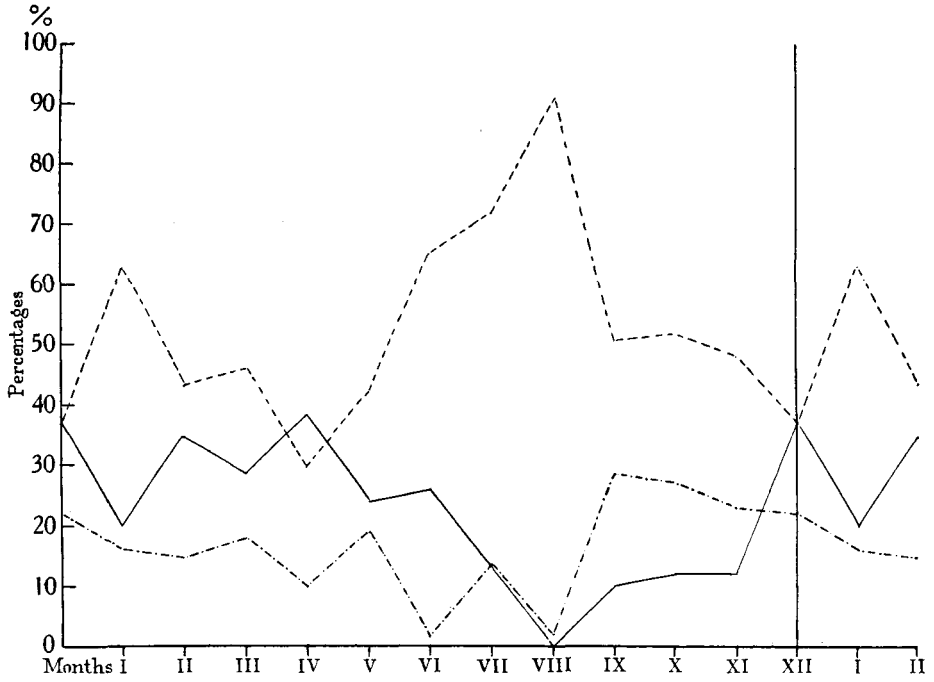
A comparison of the Böhme and the oxalic acid method of testing for indol showed that the results were fairly comparable among the 566 strains tested in both ways. On the whole, however, the oxalic acid was the slightly more sensitive reagent.

The samples were grouped under four headings according to the source of supply. The first two groups were drinking waters, namely 1622 samples from town supplies gathered from upland surface areas, and 399 from rural supplies derived from wells and springs. The last two groups, which were not potable waters, included 90 samples from swimming baths and 33 from streams, ponds and other polluted sources. 2572 coliform bacilli were isolated from town supplies, 1375 from rural supplies, 231 from swimming baths, and 119 from polluted waters. The reactions given by these cultures are recorded in Table VII (p. 50). The dominant organism in every series was *B. coli*, but there was a striking difference in the numbers of *aerogenes* present. The ratio of *B. coli* to *aerogenes* in town supplies was approximately 10 : 1, in rural supplies it was 3 : 1, in polluted ("miscellaneous") water it was 4 : 1, but in swimming baths the number of *B. coli* and *aerogenes* was almost equal. Thus the bath waters yielded a higher percentage of MR_0 organisms and a lower percentage of *B. coli* than any other series of waters.

A consideration of results on the basis of samples showed that the flora of swimming baths was much more varied with regard to coliform organisms than



Graph III. Seasonal incidence. Town water supplies. Percentages of the different types of coliform organisms among the strains isolated.



Graph IV. Seasonal incidence. Rural water supplies. Percentages of the different types of coliform organisms among the strains isolated.

– – – – – Percentage of *B. coli* (MR_+ VP_0 $indol_+$ cit_0).
——— Percentage of *B. lactis aerogenes* (MR_0 VP_+ $indol_\pm$ cit_+).
–·–·– Percentage of intermediate type (MR_+ VP_0 $indol_0$ cit_+).
I–XII denote months Jan.–Dec.

https://doi.org/10.1017/S0022172400034380 Published online by Cambridge University Press

any of the other waters. *B. coli* often occurred in combination with the other types, so that, although the percentage of *aerogenes* was very high, most samples were found to contain some *B. coli*. It is suggested that the treatment of swimming-bath waters with chlorine compounds may have caused the fall in the ratio of *B. coli* to *aerogenes* by destroying the MR_+ types more rapidly than the MR_0, but no experiments have as yet been carried out in this direction.

It is significant that if the presumptive reaction in MacConkey's medium had been considered throughout the investigation of the 2144 samples as sufficient evidence of the presence of *B. coli*, only 15 per cent. of the 1102 positive samples would have been erroneously reported to contain this organism. This suggests that the development of acid and gas in MacConkey is in itself a reliable indicator of faecal pollution, but further conclusions on this point will be withheld until the results of the soil and faecal investigations are available. The whole question of the sanitary significance of the three main coliform types can then be thoroughly discussed and the results applied to food and water analysis.

An attempt was made to detect seasonal variation in the types of bacilli found in water, and it was shown that *aerogenes* and intermediate type increased relatively in the late winter and spring months and at the same time *B. coli* began to die out. Conversely, in the summer and early autumn *B. coli* showed an increase and the citrate utilisers tended to disappear.

III. SOIL.

SOURCE OF SAMPLES.

Eighty-six soil samples were examined for the presence of coliform bacilli. These soils were collected at various seasons of the year from the moorland districts of the Pennine Range, and were carefully gathered from regions where the chances of faecal contamination appeared to be extremely slight. The area covered was an extremely wide one, the most northerly point being Longridge Fell near Preston, and the most southerly the Derbyshire "Peak District." The Pennine moorlands form a series of watersheds which are used as gathering grounds for town supplies, and many of these soils were actually taken from waterworks' ground far removed from houses and farms and unpolluted by grazing sheep. It is virtually impossible, however, to obtain a soil entirely free from pollution on account of birds and wild animals, although the samples examined here appeared to be relatively free from contamination of this kind.

The nature of the soil was generally peaty and very acid. The lowest *p*H was 4·0, only seven samples yielded a higher *p*H than 7·0, and 13 a higher *p*H than 6·0. The vegetation was usually xerophytic, consisting of the Ericaceous plants commonly found on well-drained peaty ground—*Calluna vulgaris*, *Erica tetralix* and *Vaccinium myrtillus* together with *Empetrum nigrum* and a few species of moorland grass, chiefly *Festuca ovina* and *Nardus stricta*.

All samples were collected in sterile bottles by means of sterile spoons and were taken just beneath the surface after the upper layer of soil had been scraped away. Some of them were gathered from eroded areas and others from patches of ground left exposed by uprooting the growing plants.

Technique.

Preliminary experiment showed that it was necessary to adopt an enrichment method, because the numbers of coliform bacilli were too small to give a reasonable chance of recovery if a direct plating method was used.

Standard method. 15 g. of the soil sample were ground up in a sterile mortar and enough sterile water was added to bring the whole volume up to 150 c.c. This gave a preliminary emulsion of 1 in 10 and from it four other dilutions were prepared. The presumptive *coli* test was carried out by inoculation into MacConkey's bile salt lactose peptone water. At first only the highest dilution giving acid and gas was plated out on MacConkey's agar, but later all the positive tubes were tested. Three strains were isolated in pure culture from each plate and submitted to the nine confirmatory tests already mentioned in connection with water analysis.

This method ensured that the results obtained from water and from soil would be strictly comparable, but it was found that among the soil samples there was a high percentage of false positive presumptive results. Among 13 soils examined 10 gave a positive presumptive reaction, but on subculture only six of these yielded a growth; therefore some factor which considerably reduced the viability of the organisms must have been in operation. This inhibition could have been effected by (*a*) hydrogen-ion concentration, (*b*) toxicity of the soil, (*c*) bacteriophage. Failing these, the false positive presumptive tests could have been caused by the growth of some organism other than *B. coli*.

Hydrogen-ion concentration.

The use of buffered MacConkey's medium. Experiments were carried out on the lines suggested by Thompson (1927), who found that the addition of 0·2 per cent. K_2HPO_4 in MacConkey's medium greatly reduced the number of false positive tubes which he obtained in water analysis. MacConkey's peptone water was prepared containing 1 per cent. K_2HPO_4 in double and 0·5 per cent. in single strength, and six soils were tested for *B. coli* by inoculating each one into buffered and into unbuffered medium. The percentage of false positive presumptive reactions was exactly the same in each series. It was evident that the addition of phosphate in this proportion was of no value, and higher concentrations of K_2HPO_4 merely precipitated the peptone.

Titration of soil suspension. It was found impossible to calculate the amount of $M/10$ buffer required to bring a soil suspension to pH 7·0 by the titration of the filtered extract. It then became necessary to find a suitable concentration of buffer which would maintain pH 7·0, however acid or alkaline the soil sample happened to be.

The action of buffer solutions on soil appears to depend on certain chemical and physical conditions. The most important chemical factor is the initial acidity of the soil, but the presence of clays in colloidal solution must be taken into account as well as the iron and calcium content, since both these substances are precipitated by phosphate. Also, the buffer may be adsorbed by the soil particles themselves, especially when the adsorption surface has been increased by the grinding and shaking processes.

The final concentration of buffer adopted was *M*/5, 42·984 g. Na_2HPO_4 + 10·892 g. KH_2PO_4 per litre. This was sufficient to return the majority of samples to *p*H 7·0, so that conditions, as far as acidity was concerned, were uniform in each soil suspension. Cultures of *B. coli* were then added to sterile *M*/5 buffer and to sterile water in known numbers, and MacConkey incubations were prepared from these suspensions. A comparison of the two results showed that the use of *M*/5 phosphate did not reduce the chances of recovering *B. coli*.

Five soils were examined for *B. coli* by emulsifying and diluting both in sterile water and in sterile *M*/5 phosphate buffer. The proportion of samples which produced acid and gas in MacConkey but which failed to yield a growth from the positive tubes was approximately the same in each series. Thus it was proved that the low *p*H values of the soil samples examined were not responsible for the occurrence of false positive presumptive results.

Toxicity of the soil.

A tube of double strength MacConkey inoculated with soil and giving acid and gas in 24 hours was repeatedly subcultured on agar, but no growth could be obtained. A suspension of *B. coli* was then added and plate cultures were made at first hourly, and then daily. The culture remained alive for 16 days after it was added.

Bacteriophage.

One soil was tested for bacteriophage. The results showed that this phenomenon was not the cause of the failure to isolate *B. coli* from tubes giving the presumptive test.

Anaerobes.

Having eliminated the above factors it became evident that some organism other than the *B. coli* group was responsible for the occurrence of false positive presumptive results. In the final series of experiments on technique the MacConkey tubes which gave false positives were tested for anaerobes.

The first soil to be examined in this way yielded acid and gas in ten tubes of double strength MacConkey each containing 10 c.c. of a 1 in 10 suspension of the sample. Spread plates were made from all these tubes on MacConkey's agar. Only one plate gave definitely acid colonies, two of which were isolated in pure culture and subjected to the usual confirmatory tests. Both these strains proved to be true *B. coli* (MR_+VP_0 $indol_+$ $uric_0$ cit_0). Three of the other plates yielded a few "doubtfully" acid colonies, among which there were some

organisms of the intermediate type (MR_+ VP_0 $indol_0$ $uric_0$ cit_+), but they all required 7 days for the production of gas in lactose. The remaining six tubes showed no growth even after repeated subculture. Portions of these ten MacConkey tubes were inoculated into sterile freshly boiled litmus milk, the surface of the liquid was sealed with sterile vaseline, and the cultures were incubated at 37° C. for 48 hours. Stormy fermentation was marked in eight of these tubes and Gram-positive bacilli were numerous in the films. The other two tubes gave rather doubtful results. The clot was broken up but was not absolutely typical of *B. welchii*, and there were not many Gram-positive bacilli present.

A 1 in 10 suspension of this sample of soil was prepared in sterile water, and 2 c.c. were added to each of four tubes of litmus milk, which was heated at 80° C. for 10 min. and then incubated anaerobically. A stormy clot appeared in each after 24 hours at 37° C. The remainder of the soil suspension was heated to 80° C. for 10 min., and then 10 c.c. of the heated emulsion were added to each of three tubes of double strength MacConkey. Acid and gas developed in these tubes after 48 hours' aerobic incubation at 37° C. Subcultures on MacConkey agar plates yielded no growth, but on incubating anaerobically in litmus milk a stormy clot was produced in each case and Gram-positive bacilli appeared in all films.

Another sample of soil yielded four tubes which gave false positive presumptive reactions. These tubes were all examined for *B. welchii*, and it was again proved that this organism was present and was the cause of the development of acid and gas in MacConkey's bile salt lactose medium.

Greer (1928) reports that "one of the most frequent causes of lactose fermentation in routine water analysis which is not confirmed as *B. coli*" is the growth of anaerobes, particularly *B. welchii*.

In the investigation of 86 samples of soil there were 18 in which no *B. coli* was obtained from any of the positive MacConkey tubes, and seven in which some tubes gave verifiable and others false positive presumptive reactions. Of these 25 soils 11 were not tested for anaerobes; with the other 14 samples *B. welchii* was recovered from all the tubes which gave the false positive presumptive *coli* result. It may be of interest to note here that among the 2144 water samples a false presumptive reaction was only obtained in one case; acid and gas were produced in two double strength MacConkey tubes, but on plating no growth occurred. *B. welchii* was isolated from each of these tubes.

These results indicate that the presumptive test in MacConkey was much more reliable in water than in soil analysis, but the conditions of experiment were very different in the two cases. In the soil tubes there was always a heavy deposit which provided a region of low oxygen tension and favoured the growth of *B. welchii*, while in the water cultures there was nothing to prevent diffusion of oxygen taking place more or less evenly throughout the liquid. This may not have been the only factor influencing anaerobic growth, but, whatever the causes were, it is certain that there was a very marked difference in the

significance of the presumptive *coli* test in MacConkey's medium in the two series of experiments.

RESULTS.

Results considered on the basis of strains.

Among the 86 soils examined there were 65 (75·6 per cent.) from which no coliform bacilli were obtained. The remaining 21 soils yielded 152 strains of lactose-fermenting organisms, and, since none gave irregular results, it was possible to classify them all into *B. coli*, *aerogenes* or the intermediate group. The results obtained are given in Table VIII.

Table VIII. *The classification of* 152 *organisms isolated from soil.*

Type of organism		No.	%
B. coli	(MR_+ VP_0 $indol_+$ $uric_0$ cit_0)	47	31·0
B. lact. aerog.	(MR_0 VP_+ $indol_\pm$ $uric_+$ cit_+)	4	2·6
Intermediate type	(MR_+ VP_0 $indol_0$ $uric_0$ cit_+)	101	66·4

The predominating organism was a short Gram-negative bacillus of the intermediate type, but only 12 of these strains gave positive reactions in lactose and in litmus milk within 48 hours. Among the other 89 strains there were 85 which showed delayed lactose fermentation and weak clotting ability, and four strains which required more than 5 days to produce gas in lactose, although they clotted milk after 48 hours' incubation at 37° C.

The number of *B. coli* isolated was rather less than half the number of intermediate type, and there was a striking difference between the two species in morphology and in the lactose and milk tests. The *B. coli* strains were all medium length Gram-negative bacilli which clotted milk readily and, with a single exception, produced gas in lactose within 48 hours.

The occurrence of *aerogenes* was almost negligible. This result is remarkable, since this type of coliform organism is usually considered to be typical of the soil flora, yet, in the present instance, it was only isolated four times. Every strain of *aerogenes* clotted milk within 48 hours and showed vigorous gas production in lactose.

Results considered on the basis of samples.

When the results are considered on the basis of samples, the percentages of soils yielding the various types of organisms are shown (Table IX).

Table IX. *Distribution of strains among the* 21 *positive samples.*

Number of soils which yielded:	
I. *B. coli* (MR_+ VP_0 $indol_+$ $uric_0$ cit_0)	3
II. *B. lact. aerog.* (MR_0 VP_+ $indol_\pm$ $uric_+$ cit_+)	1
III. Intermediate type (MR_+ VP_0 $indol_0$ $uric_0$ cit_+)	13
IV. *B. coli* together with the intermediate type	3
V. All three types	1
Total	21

The intermediate type was isolated from 17 (80·9 per cent.) of the 21 positive soils, and 13 of these yielded no other coliform bacillus. The occurrence of *aerogenes* was very rare, for it was only found in two soils (9·5 per cent.) and in

one of these the other two types were also present. *B. coli* existed in a larger number of samples than *aerogenes*, but it was not frequent compared with the intermediate type, for it was isolated only from 7 (33·3 per cent.).

If these figures are given as percentages, not of the 21 samples which were positive, but of the 86 soils examined, a truer picture of the soil flora is obtained as far as coliform organisms are concerned, and the relative proportions of the various types remain the same. This is shown in Table X.

Table X. *Distribution of strains among the* 86 *samples of soil examined.*

Soils giving	Number	%
I. No coliform organism	65	75·6
II. *B. coli* (MR_+ VP_0 $indol_+$ $uric_0$ cit_0)	7	8·14
III. *B. lact. aerog.* (MR_0 VP_+ $indol_\pm$ $uric_+$ cit_+)	2	2·33
IV. Intermediate type (MR_+ VP_0 $indol_0$ $uric_0$ cit_+)	17	19·76

Discussion.

There were more than 75 per cent. of the 86 samples which yielded no coliform bacilli. This proportion of unpolluted samples was very high and was probably due to the extreme care which was taken to collect virgin soils in order that the organisms isolated could be regarded as typical of the unpolluted soil flora. Other workers have obtained similar results. Houston (1898, 1901, 1903) found that *B. coli* was absent from virgin soil or was present in very small numbers, and Burke-Gaffney (1932) never succeeded in isolating coliform organisms from soil except from samples in which faecal pollution had (he supposed) taken place at some more or less remote period.

If it is true, as these results suggest, that virgin soils are relatively free from *B. coli*, then it must be assumed that most of the 86 samples examined here were free from recent contamination with excreta, and that, in the few cases where such organisms were found, pollution of this nature had actually taken place, although the occurrence of a bacillus giving all the reactions typical of the true *B. coli* group was comparatively rare.

As early as 1901 Houston recovered a number of coliform bacilli which failed to give indol and showed weak clotting ability from soil which he had previously inoculated with sewage. He believed that either the typical strains of *B. coli* ($lactose_{++}$ $milk_{++}$ $indol_+$) died out more rapidly in soil than the atypical ones, or else the truly coliform strains had lost some of their positive characters as a result of their prolonged incubation in soil. Savage (1907) found that "typical *B. coli* implanted into soil showed some alteration of character, but the changes were not extensive and no evidence was obtained that the widely aberrant organisms met with in different soils and waters ever represented typical *B. coli* altered by unfavourable environment."

More recently Kulp (1932) held 24 strains of *coli* and *aerogenes* in soil culture for a considerable period. Six strains of *coli* and two of *aerogenes* survived for 3 years and 7 months and showed at the end of that time no evi-

dence of change in the morphology, lactose fermentation, MR or VP reaction, gelatine liquefaction or citrate and uric acid utilisation, but there was a possibility that indol production might be a variable characteristic.

Hicks (1927) examined 50 strains of coliform bacilli from soil in China, among which there were 76 per cent. MR_+VP_0, 80 per cent. citrate utilisers and 68 per cent. which failed in the indol test. Burke-Gaffney (1932) obtained 24 strains of lactose fermenters from European soil of which only 4 per cent. were *aerogenes* and 58 per cent. were of the intermediate type.

On the other hand very different results have been published by other investigators, who have found that *aerogenes* is the prevailing type in soils which do not appear to be subject to faecal contamination. Johnson (1916) found 261 MR_0 strains among 363 soil cultures, and Chen and Rettger (1920) isolated 467 strains of coliform bacilli from soil of which 447 were *aerogenes*. Koser (1924, 1926, 1926 *c*) examined several series of lactose-fermenting bacilli from soil. In the first series there were 72 cultures of which half were $MR_0VP_+cit_+$, but there were also 23 MR_+VP_0 cit_+ strains and 16 of these were indol negative. In the second series 162 were subcultured of which 104 were *aerogenes*, and, in a third experiment, 104 strains from cultivated fields included 70 MR_0 VP_+cit_+ types, while 33 strains from polluted fields yielded only 11 MR_0 organisms. Raghavachari (1926) working in India isolated 518 strains of coliform bacilli from soil among which there were 484 MR_0 VP_+ cit_+ cultures, and Gray (1932) was able to isolate *aerogenes* from each of six samples of soil, apparently unpolluted, collected in Scotland, and five of these samples also yielded *B. coli*. Burke-Gaffney (1932) found that the relative proportions of the various coliform organisms were very different in soil collected in Europe and in East Africa. In the former series the intermediate type was dominant, but among 24 strains isolated from remotely polluted soils in the tropics, 67 per cent. were *aerogenes* and only 29 per cent. were the intermediate type.

In the face of such conflicting testimony regarding the natural soil flora, and in the absence of any definite evidence of change in the reactions of coliform bacilli under different conditions, the only conclusion at which it is possible to arrive is that, when such bacilli do occur in soil, the proportion of the three types varies in different areas according to the conditions which prevail there. Unfortunately in our present state of knowledge it is impossible to suggest any cause for such differences; the solution must of necessity depend upon future observations.

IV. FAECES.

SOURCE OF SAMPLES.

Thirty-four samples of human faeces were tested for the presence of different types of coliform bacilli. These samples had all been received at the laboratory for examination for pathogenic organisms, so that none of them could be regarded as normal specimens. Yet, since coliform bacilli were isolated in every case, it may be of interest to note the relative proportions of the various types of lactose fermenters which were found.

Technique.

Most of the specimens were examined by plating out on MacConkey's agar from an emulsion in peptone water, and a varying number of isolated colonies, usually five, were picked off. The number depended entirely on the appearance of the colonies; if more than one type of acid colony was observed, representatives of each were submitted to the nine tests used in water analysis.

In a few cases the primary emulsion in peptone water was inoculated into three tubes of Koser's citrate medium, one tube receiving one loopful, one receiving two loopfuls and the third receiving three loopfuls of the suspension. These cultures were incubated for 24 hours at 30° C. and then plated out on MacConkey's agar. Five or more strains of lactose-fermenting bacilli were isolated from each plate, and tested in the usual way. This method of procedure was suggested by the work of Gray (1932), and by the account of Prof. Cruikshank's technique reported in Gray's publication.

Results.

Results considered on the basis of strains.

Of the 34 samples of faeces examined there were 27 which were tested only by the direct plating method, two which were tested only by the citrate method, and five samples which were tested in both ways. The reactions of the 331 strains isolated are given in Table XI.

Table XI. *Classification of the* 331 *strains isolated from faeces.*

Method of testing	No. of samples	No. of strains isolated	*B. coli* $MR_+ VP_- indol_+ uric_- cit_-$ No.	*B. coli* %	*B. lact. aerog.* $MR_- VP_+ indol_\pm uric_+ cit_+$ No.	*B. lact. aerog.* %	Intermed. type $MR_+ VP_- indol_- uric_- cit_+$ No.	Intermed. type %	Irreg. forms No.	Irreg. forms %
Direct plating method	32	220	194	88	6	3	17	8	3	1
Citrate method	7*	111	63	57	34	31	13	12	1	1
Totals	34	331	257	78	40	12	30	9	4	1

* Five of these samples are also included among those tested by the direct method.

Among the 327 strains classified in the table 3·5 per cent. of *B. coli*, 42·5 per cent. of *aerogenes* and 33·3 per cent. of the intermediate type gave abnormal reactions in the lactose and milk tests. Eight strains of *B. coli*, eight *aerogenes* and six intermediate types produced scarcely more than a bubble of gas in lactose even after 5 days' incubation at 37° C., and the milk was clotted only on boiling. Also one *B. coli*, seven *aerogenes* and four intermediate types showed weak gas production, but clotted milk in the usual time, and two strains of *aerogenes* which readily fermented lactose failed in the clotting test.

Both series of results proved *B. coli* to be the dominant faecal organism, for 88 per cent. of the 220 strains isolated by the direct plating method, and 57 per cent. of the 111 strains isolated by the citrate method, were of this type. There was, however, a very striking difference in the relative numbers of the intermediate type and *aerogenes* when the two methods of examination were

compared. Only 8 per cent. of the 220 strains recovered by the first method were of the intermediate type and rather less than 3 per cent. were *aerogenes*, but when the citrate method was employed the percentage of the intermediate type rose to 12 and *aerogenes* increased from 3 per cent. to 31 per cent. The fact that the intermediate type was isolated more readily from direct plates than *aerogenes* probably means that this organism was present in larger numbers in the faecal suspension; on the other hand *aerogenes* seems to have grown more vigorously in citrate, for the increase in number after incubation in this medium was more marked.

Results considered on the basis of samples.

When the results of the two series of examinations are considered on the basis of samples, the same difference is evident. This is shown in Table XII.

Table XII. *Distribution of organisms among the samples.*

Method of exam.	Total samples examined	Samples giving: *B. coli* $MR_+ VP_0 indol_+ uric_0 cit_0$ No.	%	*B. lact. aerog.* $MR_0 VP_+ indol_\pm uric_+ cit_+$ No.	%	Intermed. type $MR_+ VP_0 indol_0 uric_0 cit_+$ No.	%	Irreg. forms No.	%
Direct plating	32	30	94	3	9	5	16	2	6
Citrate	7	7	100	4	57	2	29	1	14

Successful isolation by means of citrate largely depends upon inoculating the medium as lightly as possible in order to avoid adding the products of bacterial metabolism. In the present series the heavy inoculation of one, two or three loopfuls of faecal suspension in peptone water provided sufficient material to maintain the viability of *B. coli*, for this organism was isolated from every sample which had been incubated for 24 hours in citrate. At the same time the recovery of the citrate utilisers was greatly facilitated. Among the 32 samples tested by the direct plating method only 9 per cent. yielded *aerogenes*, and 16 per cent. yielded the intermediate type in contrast to 57 per cent. giving *aerogenes* and 29 per cent. giving the intermediate type among the seven samples plated out after preliminary enrichment in the citrate medium.

The five samples which were tested both by direct plating and after citrate incubation have been included in Tables XI and XII. Table XIII gives a further comparison of the two methods based on the results obtained from these samples.

It is unfortunate that so few samples were tested by each method, since it is hardly justifiable to draw conclusions from an examination of five specimens. Nevertheless the results, as far as they go, are in accord with those of Cruikshank, who is reported to have found *aerogenes* in 98 out of 135 samples of human faeces which he examined by the citrate method, and also of Gray (1932), who tested 40 specimens by a similar technique and isolated this organism in 37 cases. Taking the results of the present study as a whole based on the figures obtained from the 34 specimens, it seems justifiable to conclude that while *B. coli* is definitely the dominant organism in faeces, the intermediate type and

Table XIII. *Comparison of the results of the direct plating and the citrate method of isolation.*

Method of exam.	No. of sample	No. of strains isolated	No. of *B. coli*	No. of *B. lact. aerog.*	No. of Intermed. type	No. of Irreg. strains
Direct plating	1	15	15	—	—	—
	2	15	15	—	—	—
	3	15	15	—	—	—
	4	6	6	—	—	—
	5	15	15	—	—	—
Totals	5	66	66	0	0	0
Citrate	1	13	6	7	—	—
	2	15	15	—	—	—
	3	20	12	—	8	—
	4	18	18	—	—	—
	5	15	3	12	—	—
Totals	5	81	54	19	8	0

aerogenes also occur, although in much smaller numbers. The isolation of these types is greatly facilitated by preliminary enrichment in citrate medium, which gives the citrate utilisers a chance of development. This method, however, encourages the growth of *aerogenes* rather than the intermediate type, although both organisms are capable of activity and reproduction in the medium.

V. ICE-CREAM.

Forty-four samples of ice-cream which had been submitted to the laboratory for routine analysis were examined for different types of lactose-fermenting bacilli. These samples were collected from the Manchester district during the summers of 1931 and 1932. The method of production and the conditions of manufacture varied very considerably, but all the samples could be described as "milk products" which had been treated by heat at some time during preparation, and afterwards cooled and maintained at low temperature. The ice-cream was collected in sterile bottles from the retailer or the manufacturer, was placed in a refrigerator and immediately dispatched to the laboratory.

Technique.

Each sample was thoroughly mixed before examination. If the product was too solid for shaking it was warmed very slightly until the fluid could be shaken and pipetted with comparative ease. Five dilutions were prepared in sterile water and inoculations were made into ordinary and into double strength MacConkey for the presumptive *coli* test. Then, as in water analysis, organisms of typical morphology and staining reaction were isolated from the highest dilution giving acid and gas and subjected to the usual confirmatory tests. If none of these strains were true *B. coli* (MR_+VP_0 $indol_+$ $uric_0$ cit_0), the other positive MacConkey tubes were plated out and tested. Occasionally more than one kind of acid colony appeared on the plates, in which case representatives of all types were subcultured. The reactions given by the irregular strains were confirmed before the organisms were finally included in the series.

RESULTS.

Among the 44 samples of ice-cream there were 17 which gave no acid and gas in the MacConkey medium, the remaining 27 samples yielded 365 strains of lactose-fermenting bacilli which are classified in Table XIV.

Table XIV. *Classification of the* 365 *strains isolated from ice-cream.*

B. coli $MR_+ VP_0 indol_+ uric_0 cit_0$		*B. lact. aerog.* $MR_0 VP_+ indol_\pm uric_+ cit_+$		Intermed. type $MR_+ VP_0 indol_0 uric_0 cit_+$		Irreg. strains	
No.	%	No.	%	No.	%	No.	%
26	7	244	67	70	19	25	7

The dominant type of organism in this series of experiments was *B. lactis aerogenes* which formed 67 per cent. of the total, the intermediate type, 19 per cent., came second in order of frequency, and only 7 per cent. of the strains were *B. coli*. The number of irregular strains was very high, including twelve organisms which were $MR_+ VP_0 indol_0 uric_+ cit_+$, six organisms $MR_+ VP_0 indol_0 uric_0 cit_0$, five organisms $MR_+ VP_0 indol_+ uric_0 cit_+$, and two organisms $MR_0 VP_+ indol_0 uric_0 cit_+$.

Many of the *aerogenes* gave abnormal results in the lactose and milk tests. Twenty-one strains showed delayed lactose fermentation, six strains clotted milk only on boiling, and 13 strains gave delayed reactions in both tests. Among the 70 intermediate types 10 produced rather less than 10 per cent. of gas in the Durham tube in lactose, and five strains showed weak clotting ability as well as feeble gas production. None of the 26 *B. coli* strains gave delayed or weak reactions in either lactose or litmus milk.

The results are considered on the basis of samples in Table XV.

Table XV. *Distribution of organisms among the* 44 *samples of ice-cream.*

Samples giving							
B. coli $MR_+ VP_0 indol_+ uric_0 cit_0$		*B. lact. aerog.* $MR_0 VP_+ indol_\pm uric_+ cit_+$		Intermed. type $MR_+ VP_0 indol_0 uric_0 cit_+$		Irreg. strains	
No.	%	No.	%	No.	%	No.	%
7	26	22	82	13	48	5	19

Of the 27 samples which gave a positive presumptive *coli* result there were 22 (81·5 per cent.) which yielded *aerogenes*, and nine of these contained no other coliform bacillus. The intermediate type occurred in 48 per cent. of the samples, but *B. coli* was found only in seven (26 per cent.).

Thus in these samples of ice-cream *aerogenes* was definitely in excess of the other coliform types. This may have been because the MR_0 organism was present in large numbers in the ingredients, for milk supplies are known to contain a high percentage of *aerogenes*. Rogers, Clark and Davies (1914) found that among 124 cultures of lactose fermenters isolated from milk there were 59 which gave a gas ratio, $CO_2 : H_2$, of more than 1·5, and in some unpublished work carried out in this laboratory the proportion of MR_0 bacilli was found to be approximately 28 per cent. among 664 strains isolated from 70 samples of

milk. In one instance a "Certified" milk which repeatedly gave a positive presumptive *coli* test was never found to contain any organisms other than *aerogenes* even after continual replatings throughout a whole series of tests, and a similar result was obtained by workers at the University of Leeds in a supply of "Certified" milk from a farm near Harrogate. The persistence of this organism in such instances may possibly have been associated with a specific infection of the milk ducts or udder, but in the majority of cases where *aerogenes* is present it is probably of faecal origin. Nevertheless it is difficult to explain why the proportion of MR_0 to MR_+ should be so high in milk compared with the proportion in water, soil and faeces.

It is possible that the prolonged exposure to low temperature in the manufacture of ice-cream may have a harmful effect on *B. coli*, while allowing *aerogenes* to retain its viability. It is significant to note in this connection that the highest proportion of *aerogenes* occurred in domestic water in the late winter and spring months, and it seems probable that survival at low temperature may be another aspect of resistance on the part of this organism, which has already been observed to survive treatment with weak chlorine disinfectants such as are used in the purification of swimming-bath waters.

VI. GENERAL CONCLUSIONS.

Organisms of the coliform group are not widely distributed in nature except where faecal contamination has taken place at some period more or less remote. Most virgin soils examined in this series were free from coliform bacilli, and many of the upland surface waters contained no lactose fermenters in 100 c.c. On the other hand these organisms are contained in enormously large numbers in faeces. The dominant type is *B. coli* (MR_+VP_0 $indol_+$ $uric_0$ cit_0), but by preliminary incubation in Koser's citrate medium it has been found possible to demonstrate the presence of small numbers of *aerogenes* (MR_0VP_+ $indol_\pm$ $uric_+$ cit_+) and the intermediate type (MR_+VP_0 $indol_0$ $uric_0$ cit_+), which by the ordinary methods of isolation are usually overgrown by *B. coli*. Thus the occurrence of *aerogenes* and the intermediate type in food and water supplies may be due to faecal pollution, although the presence of these bacteria in the absence of *B. coli* would seem to suggest that the pollution had not been recent.

This fact is demonstrated in another way as far as water analysis is concerned by the results obtained in this investigation. The use of the MR, VP, indol and citrate tests proved that scarcely more than 15 per cent. of the 1102 samples giving a positive presumptive result contained no true *B. coli*. If this percentage is calculated, not on the positives, but on the total number of samples examined, the number of waters giving the presumptive test and yielding no *B. coli* is reduced to 7·84 per cent. It is doubtful then whether the use of these tests in routine analysis is justified, especially since it has been shown that *aerogenes* and the intermediate type may be recovered from faeces, and it would appear that, as far as water analysis in England is concerned, these reactions

might well be discarded, since they seem to be of more interest to the systematist than to the sanitary bacteriologist.

The incidence of *aerogenes* provided some unexpected and interesting results. It was rarely found in the soil samples examined, neither was it isolated in large numbers from stored waters, although according to the theory of longevity this bacillus would be expected to survive under such conditions longer than the other types. On the contrary it was proved to be most abundant in samples from swimming baths, all of which had been treated with a mild chlorine disinfectant, and usually contained between 0·2 and 0·5 part of chlorine per million of water. There was also an increase in the relative number of MR_6 bacilli in potable water in the late winter and spring months, a fact which can be correlated with the occurrence of *aerogenes* as the dominant coliform type in the samples of ice-cream examined. Thus it would appear that the proportions of the various types of coliform bacilli present in food and water depend on external conditions, since *aerogenes* seems to be more resistant to weak chlorine disinfectants and also to prolonged exposure to low temperature than the MR_+ organisms.

Lastly the distribution of the intermediate type has to be considered, and here the results obtained are less definite and appear to have less significance than in the case of *B. coli* and *aerogenes*. This type was dominant in soil, and was frequently isolated from ice-cream, but was rarer in water and in faeces. Burke-Gaffney (1932) also found the intermediate type dominant in European soil, but most workers have found *aerogenes* to be the dominant soil organism. It is probable that the relative proportions of the various groups of coliform organisms are governed by "local conditions" in the case of soils. Further investigation is necessary on this point.

ACKNOWLEDGMENTS.

I wish to thank Prof. H. B. Maitland for constant help and advice throughout the course of this investigation. I am also greatly indebted to Prof. W. W. C. Topley, at whose suggestion the work was started, and to Mr W. M. Shortt, M.Sc., F.I.C., for help in the soil section and for checking the results.

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(*MS. received for publication* 10. VIII. 1933.—Ed.)