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THE CLASSIFICATION OF COLIFORM BACTERIA

BY JAMES F. MALCOLM

From the West of Scotland Agricultural College

THE coliform organisms comprise a large group of lactose-fermenting, Gram-negative, rod-shaped bacteria, which are most prevalent in the intestine and in faeces but are also common in water, milk and soil. While there is considerable variation in the criteria employed by different workers for the identification of the numerous types or varieties which exist, the following characters are generally accepted as common to the group: Gram-negative, non-sporing, aerobic but facultatively anaerobic, fermenting glucose and lactose, growing well at 37° C., and saprophytic but potentially pathogenic.

These organisms are of great importance in public health. They are not only in many instances potential pathogens, but their presence in water supplies is accepted as evidence of sewage pollution, and the occurrence of large numbers in milk is regarded as an index of excessive contamination or faulty storage conditions. Further, they are a source of great economic loss in the dairy industry owing to the fact that they are frequently responsible for the spoilage of milk and the development of defects in butter and cheese.

Routine examination of water and milk for coliform bacteria by Public Health Authorities is directed at determining the incidence of these organisms, and, in the case of water, ascertaining whether they are "typical" *B. coli* (see Muir and Ritchie, 1937) or not. As a rule no attempt is made to identify particular types. On the other hand, when coliform bacteria are associated with disease conditions or with the production of specific taints in dairy produce, the recognition of particular types may be of great importance. For this purpose and also for the systematic study of the biological characters of the group, a comprehensive classification of types is essential. There is, however, considerable difference of opinion among investigators as to how coliform bacteria should be classified. For instance, the scheme of classification proposed by Mackie (1921; see also Muir & Ritchie, 1937) has little in common with that of Bergey (1934). Therefore, to anyone faced with the determination of coliform types or engaged in systematic work, the question arises as to what classification should be adopted. This difficulty was encountered by the writer in investigations (1933, 1935) of the types of coliform bacteria occurring in milk and bovine faeces, in which 1636 cultures were isolated. The present work is an outcome of the experience gained in the identification of these types and in the study of their characters.

The coliform bacteria have been the subject of much investigation since Escherich in 1885 isolated from the intestines of infants a type which he designated *B. coli communis*. Later, different types were isolated from faeces, water, milk and soil. No comprehensive classification, however, was attempted until that of MacConkey (1905, 1909). He selected a series of biological characters which he considered of most value for differential purposes, namely, the fermentation of sucrose, dulcitol, adonitol, inulin and inositol; the production of indole; the Voges-Proskauer reaction (the production of acetyl-methyl-carbinol from a fermentable sugar); liquefaction of gelatin and presence or absence of motility. He divided the organisms into four subgroups according to their sucrose and dulcitol reactions, and subdivided the subgroups into a number of types or varieties according to the other characters mentioned. MacConkey's classification provided for the differentiation of 128 types. He actually obtained thirty-six of these types with 497 strains which he isolated from various sources. Bergey & Deehan (1908) and others used MacConkey's classification. Jackson (1911) adopted a somewhat similar classification, but also made use of the raffinose, mannitol and nitrate reactions for the differentiation of types. The disadvantage of MacConkey's and similar classifications is that they lead to a multiplicity of types, the differences between which in many instances are so slight as to be of little or no significance in public health and dairy advisory work and even for systematic purposes. As the number of reactions used as differentiating criteria increases, the number of possible types increases. Thus Bergey & Deehan (1908) employed eight reactions in their classification and therefore made it possible to differentiate 256 types.

In an investigation of the characters of 630 strains of *B. coli* obtained from human faeces, Howe (1912) showed that there was no correlation between certain features, e.g. motility, fermentation of dulcitol and mannitol, indole production and nitrate reduction. Consequently, he distinguished only two types, *B. communis* (sucrose-negative) and *B. communior* (sucrose-positive). Kligler (1914) suggested that the lactose-fermenters should be subdivided according to their reactions with sucrose and salicin instead of with sucrose and dulcitol, as he found that the sucrose-salicin subgroups correlated more highly with the indole, Voges-Proskauer and gelatin reactions, than did the sucrose-dulcitol subgroups. Prescott & Winslow (1915), like Howe (1912), were of the opinion that the correlation of characters should be taken into consideration, and that coliform organisms should be classified on a statistical basis.

Rogers and his co-workers (1914–18) in an examination of the characters of coliform strains from faeces, milk and grains, confirmed the findings of Smith (1890, 1893, 1895), Russell & Bassett (1899), Harden (1901, 1905) and others as to the value for the differentiation of coliform types of the ratio of CO_2 to H_2 in their fermentation products. Rogers and his associates distinguished two groups of coliform organisms, (1) a low-ratio group consisting

of strains which gave a CO_2 to H_2 ratio of 1·06, e.g. *B. coli*, and (2) a high-ratio group consisting of strains which gave a CO_2 to H_2 ratio ranging from 1·90 up to 3·00, e.g. *B. aerogenes*. These workers found that there was a close correlation between the gas ratio of cultures of coliform bacteria and their source. Clark & Lubs (1915) showed that the low-ratio group could be distinguished from the high-ratio group by their acid-producing powers, cultures of the low-ratio group producing under certain conditions in carbohydrate media a much higher acidity than cultures of the high-ratio group. They found that the difference in degree of acidity could be readily determined by means of the indicator, methyl red, and introduced the methyl-red test for distinguishing low- from high-ratio coliform types. Levine (1916*a*, *b*, *c*), Johnson (1916), Hulton (1916), Johnson & Levine (1917), Levine (1918), Rogers *et al.* (1918) and others noted that there was an almost perfect inverse correlation between the methyl-red and Voges-Proskauer reactions of coliform bacteria. Thus the low-ratio group was methyl-red-positive and Voges-Proskauer-negative; and the high-ratio group, methyl-red-negative and Voges-Proskauer-positive.

Levine (1918) in a statistical examination of the characters of coliform organisms concluded that the group should be subdivided on correlated characters. He elaborated a scheme of classification whereby the lactose-fermenters were subdivided, according to the methyl-red, Voges-Proskauer and starch reactions, into an *aerogenes-cloacae* group (either methyl-red-negative and Voges-Proskauer-positive, or starch-positive) and a *coli* group (methyl-red-positive, Voges-Proskauer-negative and starch-negative). These subgroups were then subdivided according to various biochemical reactions and motility, the correlation of characters being again taken into consideration.

Mackie (1921) suggested that the inositol and indole reactions of coliform organisms were important criteria for their classification. He considered that the fermentation of lactose and other carbohydrates was of less significance for differential purposes, and suggested that all the glucose-fermenting saprophytic members of the coli-typhoid group should be recognized as coliform bacteria, irrespective of the nature of their other fermentative reactions. Thus he included in the coliform group certain non-proteolytic glucose-fermenting saprophytes, which resemble *B. coli* in their general characters, but which do not ferment lactose or do so only after mutation, e.g. the "paracolon" bacilli. Mackie found that strains having the characters common to this coliform group did not liquefy gelatin, apart from strains which might be classified as *Proteus*.[1] He pointed out that inositol fermentation is correlated with other characters, e.g. non-motility, encapsulation,

[1] In testing the proteolytic properties of his strains, Mackie kept gelatin stab cultures at 22° C. for only 2 weeks. In this period few of the strains of the gelatin-liquefying types included by MacConkey and others in the coliform group, e.g. *B. cloacae* and *B. oxytocus perniciosus*, would give positive reactions. These strains may take from 4 to 12 weeks or more to liquefy gelatin at 22° C. and even at 30 and 37° C.

production of large, thick, opaque, slimy colonies, and fermentation of lactose, adonitol, sucrose, raffinose and salicin. He classified the glucose-fermenters of the coli-typhoid group into the following subgroups:

A. Gas-producing, indole-forming, non-inositol-fermenting and non-liquefying, e.g. "typical" *B. coli.*

B. Gas-producing, non-indole-forming, non-inositol-fermenting, and non-liquefying. No named species belongs to this subgroup if slow liquefying types, e.g. *B. cloacae*, are excluded.

C. Gas-producing, inositol-fermenting, non-liquefying, e.g. *B. lactis aerogenes.*

D. Non-gas-producing in all the sugars fermented and non-liquefying, e.g. *B. coli anaerogenes.*

Koser (1924) found that *aerogenes-cloacae* types (methyl-red-negative, Voges-Proskauer-positive) were capable of utilizing the citrate radicle as the sole source of carbon, while *B. coli* types (methyl-red-positive, Voges-Proskauer-negative) of faecal origin were unable to do so. Consequently, the former grew readily in a synthetic citrate medium (a positive Koser reaction) while the latter did not develop (a negative Koser reaction). However, he found that in some instances organisms resembling *B. coli* (methyl-red-positive, Voges-Proskauer-negative), but of soil origin, could attack the citrate radicle and give a positive Koser reaction.

The system of classification employed by Bergey (1923, 1934) is almost entirely different from that of Mackie. He divides the lactose-fermenters into Voges-Proskauer-negative types, genus *Escherichia*; and Voges-Proskauer-positive types, genus *Aerobacter*. The *Escherichia* are subdivided into twenty-two species according to sucrose fermentation, gelatin liquefaction, motility, salicin and dulcitol fermentations, action on milk and nitrate reduction. The *Aerobacter* are subdivided into seven species according to motility, sucrose and dulcitol fermentations, gelatin liquefaction and gas formation at 37° C. Owing to the fact that the Voges-Proskauer reaction shows an almost complete inverse correlation with the methyl-red reaction, it also correlates very closely with the gas ratio of coliform types. Consequently, the genus *Escherichia* consists of methyl-red-positive and low-ratio types, and the genus *Aerobacter* of methyl-red-negative and high-ratio types.

The classification of Bergey, however, is not satisfactory for reasons which may be stated briefly as follows:

(1) The coliform group of bacteria consists of a gradation of types, so closely linked together as to render it undesirable to divide the group into two genera. Thus although the *Escherichia* types of Bergey differ from the *Aerobacter* in their manner of carrying out fermentation, nevertheless, with regard to other characters the former frequently present identical features to the latter, as in *B. friedländeri* (*Escherichia* types) and *B. aerogenes* (*Aerobacter* types).

(2) The inositol and indole reactions have not been employed as differential criteria in spite of the fact that, as shown by Mackie (1921) and the writer (1933, 1935), they are highly characteristic properties of various important subgroups of coliform bacteria. Further, the importance of the inositol reaction as a differential is evident from the findings of Hay (1932) in a study of the *B. mucosus capsulatus* group (inositol-fermenting organisms closely allied to, if not identical with, *B. lactis aerogenes*).

(3) The gelatin reaction is not satisfactory for differentiating types, as those coliform organisms which liquefy gelatin do so as a rule very slowly, the process taking perhaps from 4 to 12 weeks at 20° C. and even at 30 and 37° C. The test is therefore of little value in the routine analysis of water, etc. In addition, a fairly high proportion of strains otherwise of classic gelatin-liquefying types, e.g. *B. cloacae* (MacConkey's type no. 108) and *B. oxytocus perniciosus* (MacConkey's type no. 65), fail to liquefy gelatin even after prolonged incubation (Malcolm, 1933, 1935). Also, misleading results may be obtained under such conditions owing to the liquefaction being due merely to the action of endo-proteases which have been liberated from the dead bacterial cells (Committee on Bacteriological Technic of the Society of American Bacteriologists, 1934). In any case, gelatin-liquefying strains of coliform bacteria can usually be identified by other properties (see later). Bergey in the Key to the genera of tribe Bacterieae notes that gelatin is not liquefied by the members of the genus *Escherichia*, but includes in the genus a number of liquefying species.

(4) In Bergey's classification the salicin reaction has been used for differentiating species of *Escherichia*. The writer (1933), however, found that different strains of even the classic types, e.g. *B. coli communis* Escherich and *B. coli communior*, varied in their salicin reactions. Thus with ninety-five cultures of *B. coli communis* Escherich, obtained from milk, sixty-nine gave positive reactions in salicin and twenty-six gave negative. With 193 cultures of *B. coli communior*, 181 gave positive reactions and twelve gave negative. Mackie (1921) obtained similar results with strains of *B. coli communis* Escherich, *B. coli communior* and other types isolated from human faeces and from other sources.

(5) Bergey's subdivision of the non-motile, Voges-Proskauer-positive types according to the sucrose and dulcitol fermentations is not satisfactory, as it includes in the species *Aerobacter aerogenes* certain non-dulcitol-fermenting types which produce indole and liquefy gelatin. These types, except for their dulcitol reactions, are very similar to *B. oxytocus* types, and should be included in the same species, namely, *Aerobacter oxytocum*. On the other hand, in the case of non-motile Voges-Proskauer-positive types indole production, unlike dulcitol fermentation, correlates fairly closely with liquefaction of gelatin and therefore is of more value for differential purposes.

Werkman & Gillen (1932) suggested that certain coliform types which produced trimethylene glycol should be placed in a separate genus, termed

Citrobacter. These types were methyl-red-positive, Voges-Proskauer-negative and Koser-positive and therefore were intermediate between the *B. coli* and *B. aerogenes* types. Levine and his co-workers (1932) found that intermediate coliform types obtained from eggs differed from other coliform types in producing sulphuretted hydrogen from Difco proteose peptone, but did not know whether the former produced trimethylene glycol. In all instances their intermediate strains failed to produce indole. On the other hand, Tittsler & Sandholzer (1935) showed that intermediate coliform types varied with regard to Koser reaction, sulphuretted hydrogen and indole production and cellobiose and α-methylglucoside fermentation, and concluded that intermediate coliform types were so heterogeneous in character that their inclusion in a separate genus was hardly justified.

It is evident from a comparison of Bergey's classification with that of Mackie and others, that there is a wide divergence of opinions as to the classification of coliform bacteria. In the various systems of classification which have been used, differences occur, not only in the criteria employed for differential purposes, but, where the same criteria have been adopted, in the value attached to them.

In the writer's investigations (1933, 1935) of the coliform flora of milk and bovine faeces, 797 cultures of lactose-fermenting coliform bacteria were isolated from milk and 839 from faeces. This figure includes 244 cultures obtained from bovine faeces during preliminary experimental work. The cultures were typed according to MacConkey's criteria and Koser's citrate reaction. The types from milk were grouped according to the Koser, indole, Voges-Proskauer and gelatin reactions, but as regards those from faeces, the inositol reaction was substituted for the gelatin for reasons which have been already mentioned. The latter method of grouping, however, was regarded merely as a provisional and tentative arrangement until such a time as it was possible to make a more detailed study of the characters of all the cultures isolated. Accordingly the following observations on the classification of coliform bacteria are based on a study of the characters of 1636 cultures obtained from milk and bovine faeces.

The identification of types

All the cultures under examination had the following characters: Gram-negative, non-sporing and rod-shaped; capable of growing under aerobic conditions and at 37° C.; fermenting glucose and lactose with formation of acid and gas. The following additional characters were determined: fermentation of sucrose, dulcitol, adonitol, inulin, mannitol, inositol, raffinose and salicin; production of indole; growth in Koser's citrate medium; Voges-Proskauer and methyl-red reactions; liquefaction of gelatin; and, in many instances, encapsulation. The reactions in maltose, fructose, galactose, glycerol and dextrin were not determined because, as shown by Mackie (1921), these substances are fermented as a rule by all lactose-fermenting coliform bacteria.

METHODS

Stained smears were made from agar-slope cultures to find out whether the organisms were Gram-negative, non-sporing rods. The tests to determine the presence or absence of motility and the biochemical reactions were then carried out as follows:

(1) *Motility.* Cultures in peptone-water medium were kept at room temperature and examined for motility at 24 and 48 hr. Much better results were obtained by this method than by incubating the cultures at 37° C. and testing for motility at 6, 8 and 24 hr. It was found that the organisms were generally more actively motile at room temperature. Cultures which appeared to be non-motile were retested at least twice, fresh cultures being prepared for this purpose.

(2) *Encapsulation.* Cultures in broth or on agar were examined after 1–4 days' incubation by the method recommended by Howie & Kirkpatrick (1934). The organisms in the broth culture or in a suspension in broth from the agar-slope or plate culture were stained with dilute carbol-fuchsin. Eosin solution was now added to provide the background for the demonstration of capsules by negative staining. Then a film preparation was made and allowed to dry. The slide was examined under the oil-immersion lens. Good results were also obtained by a similar procedure, but employing rose bengal (1% aqueous solution) instead of carbol-fuchsin; and nigrosine (1% aqueous solution) instead of eosin solution. This method is recommended by Maneval (1934). If difficulty was experienced in staining the bodies of the cells, a dry, but unfixed, smear was made from the culture or suspension and the rose-bengal and nigrosine solutions were then applied as before.

(3) *Fermentation of glucose*, etc. The fermentation tests were carried out at 37° C. by means of Durham's fermentation tubes, the medium consisting of 1% peptone water with Andrade's indicator and 0·5% of the fermentable substance. In the case of lactose, MacConkey's lactose bile salt broth was used. Incubation was continued for 14 days.

(4) *Production of indole.* Cultures in tubes containing 4 or 5 ml. peptone (Witte's or Fairchild's) water medium were incubated at 37° C. for 10 days and then tested for indole by means of Ehrlich's test. Good results were also obtained by the use of the indole reagent employed by Kovács (1928). This reagent consists of 5 g. *p*-dimethyl-amino-benzaldehyde, 75 ml. amyl alcohol and 25 ml. concentrated HCl. Approximately 0·4 ml. of the reagent was added to each culture, the tube being shaken and allowed to stand for a few minutes before the observation was made.

(5) *The Koser-citrate reaction.* Cultures in Koser's fluid citrate medium were incubated at 37° C., a positive reaction being indicated by the presence of marked turbidity within 7 days. If the reaction was negative or there was only a faint turbidity, the test was repeated in duplicate.

(6) *The methyl-red and Voges-Proskauer reactions.* Cultures in a peptone-water medium containing 0·5% peptone, 0·5% glucose and 0·5% dipotassium hydrogen phosphate, were incubated at 37° C. for 3 days and then tested for methyl-red and Voges-Proskauer reactions. If the methyl-red and Voges-Proskauer reactions did not correlate inversely, repeat tests were made with subcultures 24, 48 and 96 hr. old.

It has been shown by Linton (1924), Georgia & Morales (1926), Paine (1927), Williams & Morrow (1928) and Ruchhoft *et al.* (1931) that the Voges-Proskauer test may give anomalous results owing to the fact that with certain Voges-Proskauer-positive types acetyl-methyl-carbinol is merely an intermediate product of glucose fermentation and cultures vary with regard to the stage of incubation at which the presence of this substance can be detected. It is therefore not possible to fix a definite period at which the test should be made, the best procedure in practice with cultures giving doubtful reactions being to make repeat tests with subcultures of various ages, as stated above.

(7) *Liquefaction of gelatin.* Two tubes of gelatin medium were heavily inoculated from an agar-slope culture and capped to prevent evaporation. The one subculture was kept at 37° C. for 12 weeks, the tube being examined at intervals for digestion by placing it in cold water for a few hours to permit undigested gelatin to solidify. If the gelatin failed to solidify, the tube was kept for a further 24 hr. at room temperature before the reaction was recorded. The other subculture was kept at room temperature for 8 weeks, and if the gelatin was still not digested it was then incubated at 37° C. for 4 weeks.

Important coliform subgroups and their distinguishing characters

Although there is a very large number of different types of coliform bacteria, the vast majority of these types can be included in a few subgroups, each of which is characterized by an assemblage of outstanding features. The subgroups most commonly recognized are the *B. coli* or "typical" *B. coli*, the *B. aerogenes*, the *B. cloacae* and the *B. oxytocus*.

The *B. coli* types are the most prevalent organisms in the intestines and in faeces. They produce a relatively high acidity in carbohydrate media, giving methyl-red-positive reactions; and form a moderate amount of gas, the CO_2 to H_2 ratio of which is low, varying from 1 to 0·5. They do not produce acetyl-methyl-carbinol (negative Voges-Proskauer reaction). They ferment glucose, fructose, galactose, mannitol, arabinose, maltose and lactose, but attack less frequently sucrose, raffinose, dulcitol and salicin, and in only a comparatively small number of instances, adonitol. They all fail to ferment inositol, inulin and starch, and cannot obtain their carbon from citrates (negative Koser-citrate reaction) or their nitrogen from uric acid. They attack tryptophane with production of indole, but do not liquefy gelatin. They are non-encapsulated, do not produce raised mucoid colonies, and are most frequently motile.

The *B. aerogenes* types carry out fermentation in a different manner to *B. coli*. Thus they produce a low acidity in carbohydrate media, giving methyl-

red-negative reactions, but form a relatively large amount of gas, the CO_2 to H_2 ratio of which is high, varying from 2 to 3. They also produce acetyl-methyl-carbinol (positive Voges-Proskauer reaction). They have much higher fermentative powers than the *B. coli* types, attacking in all instances a wide range of substances, including not only those substances usually fermented by the *B. coli* but also inositol and adonitol. They almost always ferment salicin, sucrose and raffinose; many can attack inulin, and some even starch. Further, they can utilize citrate as a source of carbon (positive Koser-citrate reaction) and uric acid as a source of nitrogen. They do not produce indole and do not liquefy gelatin. They are frequently encapsulated and as a rule non-motile. They grow very strongly on artificial media, forming colonies which are larger and thicker than those of *B. coli* and are in many instances mucoid.

The *B. cloacae* types produce a low acidity in carbohydrate media (negative methyl-red reaction), but a relatively large amount of gas. The CO_2 to H_2 ratio is high, varying from 2 to 3. They are Voges-Proskauer-positive. They have not so wide fermentative powers as *B. aerogenes*, failing to ferment inositol and adonitol. However, they can utilize citrate as a source of carbon (positive Koser-citrate reaction) and obtain their nitrogen from uric acid. They do not produce indole, but are capable of causing slow liquefaction of gelatin. They are motile, frequently encapsulated and produce large, thick mucoid colonies. The *B. oxytocus* types produce a low acidity in carbohydrate media (negative methyl-red reaction), but a large amount of gas. The CO_2 to H_2 ratio is high, varying from 2 to 3. They are Voges-Proskauer-positive, and have wide fermentative powers, even wider than the *B. aerogenes*. Not only do they ferment in all instances inositol, adonitol, sucrose, raffinose, and salicin, but they generally ferment dulcitol and inulin. They can obtain their carbon from citrates (positive Koser-citrate reaction) and their nitrogen from uric acid. They produce indole from tryptophane and can slowly liquefy gelatin. They are non-motile, are frequently encapsulated and produce large, thick, mucoid colonies.

It is evident that there is a well-marked contrast between the *B. coli* and *B. aerogenes* types with regard to acid-producing powers, as indicated by the methyl-red reaction; amount of gas formed; ratio of CO_2 to H_2; Voges-Proskauer, Koser, inositol and indole reactions; motility; encapsulation and type of colony. On the other hand, types belonging to the *B. cloacae* and *B. oxytocus* subgroups are intermediate in character. These four subgroups can be readily distinguished from one another by the following important characters: Voges-Proskauer reaction, Koser reaction, inositol fermentation and indole production. It is not necessary to include the gas ratio and methyl-red reaction, as there is an almost complete correlation between these features and the Voges-Proskauer reaction. If the Voges-Proskauer, Koser, inositol and indole reactions are used as differentiating criteria, it is possible to subdivide the coliform group into eight subgroups, each consisting of a number of MacConkey and other types (see Table I).

Table I. *Showing biological characters of* 1597 *cultures of coliform bacteria obtained from milk and bovine faeces by ordinary and by enrichment methods of isolation*

Subgroups of types	$CO_2 : H_2$	Voges-Proskauer	Koser	Inositol	Indole	MacConkey's types	Motility		Adonitol		Sucrose		Dulcitol		Inulin		Gelatin		Total
							+	−	+	−	+	−	+	−	+	−	+	−	
1. *B. coli*	Low	−	−	−	+	1, 2, 4, 5, 33, 34, 35, 71, 72, 100, 106, 107	785	97	106	776	512	370	692	190	0	882	5	877	882
2. Intermediate	,,	−	+	−	+	As above but Koser-positive	13	5	7	11	12	6	12	6	0	18	1	17	18
3. Intermediate	,,	−	+	−	−	7, 36, 70, 74, 109	124	2	1	125	48	78	40	86	0	126	9	117	126
4. Intermediate	,,	−	+	+	+	66,[1] 101	1	22	23	0	23	0	7	16	1	22	0	23	23
5. *B. friedländeri*	,,	−	+	+	−	68, 99, 104[1]	4	29	33	0	33	0	14	19	5	28	3	30	33
6. *B. cloacae*	High	+	+	−	−	3, 69, 73, 75,[1] 105, 108	198	13	4	207	211	0	59	152	13	198	141	70	211
7. *B. oxytocus*	,,	+	+	+	+	65, 97[1]	4	67	70	1	71	0	55	16	59	12	47	24	71
8. *B. aerogenes*	,,	+	+	+	−	67, 98, 102, 103	34	199	222	11	232	1	42	191	43	190	24	209	233

(The mannitol, raffinose, salicin and methyl-red reactions have not been included for reasons given in text.)

[1] Differing from MacConkey's type in inositol reactions. Thirty-nine cultures out of a total of 1636 were of atypical strains and are not included in the table. Details of their characters are given in text.

The biological characters of the subgroups

Subgroup 1 (B. coli *types*)*: Voges-Proskauer-negative, Koser-negative, inositol-negative, indole-positive.* The organisms of this subgroup are commonly termed the "typical" *B. coli* and include MacConkey's types, nos. 1, 2 (*B. acidi lactici* Hüppe, 4 (*B. grünthal*), 5 (*B. vesiculosus*), 33, 34 (*B. coli communis* Escherich or *Escherichia coli*), 35 (*B. schafferi*), 71 (*B. coli communior* or *Escherichia communior*), 72 (*B. neapolitanus*), 100, 106 and 107. The prototype is *B. coli communis* Escherich (MacConkey's type no. 34). The cultures of this group gave methyl-red-positive reactions. They did not produce raised, mucoid colonies and did not become encapsulated. The majority (785 out of 882) were motile (see Table I); the non-motile cultures were chiefly of MacConkey's types nos. 2, 5, 35, 72 and 107. Most of the cultures (776 out of 882) gave negative reactions in adonitol. The 106 adonitol-positive cultures were almost the only coliform cultures which failed to show a direct correlation between the inositol and adonitol reactions. The cultures varied widely with regard to sucrose and dulcitol reactions and no significance could therefore be attached to these features as group criteria, but they were of value for the differentiation of types. None of the cultures fermented inulin, and with rare exceptions they failed to liquefy gelatin within 3 months. Some of the intermediate cultures (fermenting cellobiose or producing H_2S) of Tittsler and Sandholzer apparently belonged to this subgroup.

The types belonging to this subgroup are by far the most prevalent organisms in the intestines, faeces and sewage, and if ordinary methods of isolation are employed, they include the bulk of the coliform cultures obtained from such sources (see Table II). They are also the most prevalent forms in milk (see Table II), their presence being due to direct or indirect contamination with faeces (Malcolm, 1933). While 882 of the 1636 cultures isolated from milk and bovine faeces (see Table I) were *B. coli* types, the number would have been much greater but for the fact that with the last hundred faecal specimens, methods of isolation were employed suitable for the enrichment of Koser-positive types, and all *B. coli* cultures obtained from these specimens were discarded.

Subgroup 2 (*Koser-positive* B. coli)*: Voges-Proskauer-negative, Koser-positive, inositol-negative and indole-positive.* These types are similar in all respects to *B. coli* types apart from the fact that they are Koser-positive. Koser (1924) obtained Voges-Proskauer-negative, Koser-positive, indole-positive types from soil, but rarely from faeces. Ruchhoft *et al.* (1931) found them occasionally in surface waters and thought that they were of soil rather than of faecal origin. These investigators did not classify their cultures according to the inositol reaction, but Koser's cultures of these types were in all instances adonitol-negative and therefore must have belonged to this subgroup. Hay (1932) also reports the occurrence of such Koser-positive

Table II. *Types of coliform bacteria isolated*

Subgroups of types	Voges-Proskauer	Koser	Inositol	Indole	By ordinary methods: Milk	By ordinary methods: Bovine faeces	By enrichment methods: Bovine faeces
1. *B. coli*	−	−	−	+	435	447[1]	All cultures discarded
2. Intermediate	−	+	−	+	1	0	17
3. Intermediate	−	+	−	−	74	2	50
4. Intermediate	−	+	+	+	13	0	10
5. *B. friedländeri*	−	+	+	−	20	1	12
6. *B. cloacae*	+	+	−	−	94	2	115
7. *B. oxytocus*	+	+	+	+	22	3	46
8. *B. aerogenes*	+	+	+	−	105	4	124
					764	459	374

[1] 117 of these cultures were obtained from bovine faeces during preliminary experimental work on enrichment methods.

B. coli types, and some of the intermediate cultures (cellobiose-positive as well as cellobiose-negative) of Tittsler & Sandholzer (1935) appear to have belonged to this subgroup.

It will be seen from Table I that only eighteen cultures of this subgroup were isolated; one of these was from milk and seventeen from bovine faeces. The faecal cultures were obtained by brilliant green and other enrichment methods of isolation (Malcolm, 1935). It would therefore appear that such types are of comparatively rare occurrence in faeces and in milk. This subgroup may be regarded as particularly hardy *B. coli* types, which are highly resistant to brilliant green and can grow sufficiently well in Koser's citrate medium to give a distinct turbidity (a positive reaction). This variation from the *B. coli* types is of less significance when it is borne in mind that, as shown by Ruchhoft *et al.* (1931), even the typical *B. coli* grow slightly in the citrate medium. Moreover, a large number of the cultures, which were obtained from faeces and which have been included in this subgroup, after subculturing for several years in artificial media gave Koser-negative reactions, in other words they were similar to the typical *B. coli*.

Subgroup 3: Voges-Proskauer-negative, Koser-positive, inositol-negative and indole-negative. This subgroup includes MacConkey's types nos. 7, 36, 70, 74 and 109. These types resemble the *B. coli* with regard to their Voges-Proskauer, methyl-red, inositol, adonitol, inulin and gelatin reactions, and also in the fact that in almost all instances they are motile, but they differ from them in being Koser-positive and indole-negative, and, in some instances, in producing large mucoid colonies and in becoming encapsulated. Like the *B. coli*, they vary widely in their sucrose and dulcitol reactions (see Table I).

Koser (1924, 1926) frequently obtained Voges-Proskauer-negative, Koser-positive, indole-negative cultures from the soil, but rarely from faeces.

Ruchhoft *et al.* (1931) found that they were of extremely rare occurrence in human and animal faeces. They have also been isolated from water by Bardsley (1926), Lewis & Pittman (1928), Ruchhoft *et al.* (1931) and others. Burke-Gaffney (1932) showed that they occurred more frequently than other coliform types in samples of soil collected in Europe, but less frequently than *B. aerogenes* types in soil from East Africa. Bardsley (1934) found that they were the most prevalent types of coliform bacteria in soil, but their incidence was lower in water and faeces. Some of the *Citrobacter* (or trimethylene-glycol-producing) types of Werkman & Gillen (1932) and in all likelihood many of the intermediate types of Levine *et al.* (1932) and those of Tittsler & Sandholzer (1935) belonged to this subgroup. As the inositol and adonitol reactions are in some instances not recorded by the above-mentioned workers, it is not always possible to determine whether their cultures belonged to this subgroup or subgroup 5.

Altogether 126 cultures of subgroup 3 were obtained by the writer, seventy-four from milk and fifty-two from bovine faeces. Cultures were isolated from only two specimens of bovine faeces by the ordinary methods, but they were obtained from approximately one-seventh of the specimens when methods suitable for the enrichment of Koser-positive organisms were employed. It would therefore appear that such bacteria are occasionally present in bovine faeces, but only in small numbers.

Subgroup 4: Voges-Proskauer-negative, Koser-positive, inositol-positive and indole-positive. This subgroup includes MacConkey's type no. 101 and another type which is similar in all respects to MacConkey's type no. 66 apart from the fact that it is inositol-positive. (MacConkey's type no. 66 is peculiar in that the adonitol reaction, being positive, does not correlate with the inositol reaction.) Six cultures of the modified or inositol-positive type no. 66 were obtained from milk and bovine faeces, while only one culture was isolated of MacConkey's type no. 66. The latter has been included in subgroup 2. Mackie (1921) obtained three cultures of this modified type no. 66 from human faeces. He did not obtain any cultures of typical no. 66. While the cultures of subgroup 4 resemble the *B. coli* subgroup in being low-ratio types, giving Voges-Proskauer-negative and methyl-red-positive reactions, and in producing indole, they are similar to the *B. aerogenes* subgroup in their Koser, inositol, adonitol and sucrose reactions, and in the fact that they are almost always non-motile and in many instances produce large mucoid colonies and show encapsulation. Werkman & Gillen (1932) found that certain types apparently belonging to this subgroup produced trimethylene glycol and included these types in the genus *Citrobacter*.

Only twenty-three cultures of types of subgroup 4 were obtained by the writer, thirteen from milk and ten from bovine faeces. All the faecal cultures were isolated by enrichment methods. Mackie (1921) obtained only seven of such cultures from human faeces and one from urine out of a total of 246 cultures isolated from various sources. Koser (1924) did not obtain any of

these types from the soil, his Voges-Proskauer-negative, Koser-positive, indole-positive cultures being in all cases adonitol-negative. It would therefore appear that types of subgroup 4 are of comparatively rare occurrence in milk, faeces, soil and water.

Subgroup 5 (B. friedländeri *types*): *Voges-Proskauer-negative, Koser-positive, inositol-positive and indole-negative.* This subgroup includes MacConkey's types nos. 68 (*B. friedländeri*), 99 and 104.[1] These organisms are intermediate in character between the *B. coli* and the *B. aerogenes* types. Thus they are similar to the *B. coli* in that they are low-ratio types, and give Voges-Proskauer-negative and methyl-red-positive reactions, but they resemble closely the *B. aerogenes* types in their Koser, inositol, indole, adonitol and sucrose reactions and, in the fact, that they are, as a rule, non-motile, produce large, thick, mucoid colonies and show encapsulation. With few exceptions they fail to liquefy gelatin. Only thirty-three cultures of these types were isolated, twenty being from milk and thirteen from faeces. With one exception, the faecal cultures were obtained by enrichment methods of isolation. Mackie (1921) and Hay (1932) have obtained strains of this subgroup from human faeces. It would therefore appear that such organisms occur to a limited extent in faeces. *B. friedländeri* is said to be found particularly in the upper respiratory tract, whence it may find its way to the intestines, but capsulated coliform organisms found in the nose or nasopharynx may give the biological reactions of typical *B. aerogenes.*

Subgroup 6 (B. cloacae *types*): *Voges-Proskauer-positive, Koser-positive, inositol-negative and indole-negative.* This subgroup includes MacConkey's types nos. 3, 69, 73, 75,[1] 105 and 108, the prototype being no. 108 (*B. cloacae* or *Aerobacter cloacae*). The organisms of this subgroup are intermediate in character between the *B. coli* and the *B. aerogenes* types. Like the *B. aerogenes* they are high-gas-ratio types. They also resemble them in their Voges-Proskauer, methyl-red, Koser, indole and sucrose reactions, and in their ability to live under natural conditions in the soil and in water, and in their resistance to brilliant green. Further, they frequently produce large, mucoid colonies and become encapsulated. They are, however, similar to the *B. coli* types in their inositol and adonitol reactions and in the fact that in most instances they are motile. They are peculiar in that they frequently liquefy gelatin. While *B. cloacae* types are Koser-positive, Hay (1932) found that they do not grow so strongly on Simmons' citrate agar as *B. aerogenes* types, and therefore they apparently do not use the citrate so readily as the latter.

The *B. cloacae* types are commonly regarded as non-faecal organisms, as in numerous investigations they have been rarely obtained from faeces but frequently from the soil, water, plants and grain, and contaminated milk. Of the total number of cultures isolated (see Table I), 211 belonged to this subgroup; ninety-four of these cultures were obtained from milk and 117 from bovine faeces. Almost all the cultures from the faecal specimens were obtained by enrichment methods of isolation (see Table II). It would therefore appear

[1] Differing from MacConkey's type in inositol reaction.

that *B. cloacae* types occur in small numbers in bovine faeces but are very frequently present. This is in accordance with the results obtained for human faeces by Cruickshank & Cruickshank (1931) and Hay (1932), who have shown by enrichment methods that such organisms are present in small numbers in most specimens of human faeces. The organisms of this subgroup cannot therefore be regarded as non-faecal types.

Subgroup 7 (B. oxytocus *types*): *Voges-Proskauer-positive, Koser-positive, inositol-positive and indole-positive.* This subgroup includes MacConkey's types nos. 65 (*B. oxytocus perniciosus* or *Aerobacter oxytocum*) and 97.[1] They are high-gas-ratio types and give methyl-red-negative reactions. They produce large, thick mucoid colonies and are frequently encapsulated. They may be considered to be indole-positive *B. aerogenes* types (Jordan, 1928; Ruchhoft *et al.* 1931), but they have as a rule greater fermentative powers than *B. aerogenes* and in many instances slowly liquefy gelatin. They are more closely allied to *B. aerogenes* than to *B. cloacae* as shown by the fact that they are inositol-fermenters, and as a rule non-motile. It will be seen from Table I that with few exceptions the cultures of this subgroup fermented adonitol, sucrose, dulcitol and even inulin. No other subgroup contained so high a proportion of inulin-fermenters. The majority of the cultures also slowly liquefied gelatin. Seventy-one cultures of this subgroup were isolated; twenty-two were obtained from milk and forty-nine from bovine faeces. The faecal cultures, with three exceptions, were obtained by enrichment methods. It is therefore evident that these organisms are frequently present in bovine faeces, but only in small numbers. This is in accordance with the results obtained for human faeces by Cruickshank & Cruickshank (1931) and Hay (1932).

Subgroup 8 (B. aerogenes *types*): *Voges-Proskauer-positive, Koser-positive, inositol-positive and indole-negative.* This subgroup includes MacConkey's types nos. 67, 98, 102 and 103, the prototype being no. 103 (*B. lactis aerogenes* or *Aerobacter aerogenes*). The organisms of this subgroup are high-gas-ratio types, giving methyl-red-negative reactions. They are typical inositol-fermenters, being encapsulated, usually non-motile, and forming large, thick, mucoid colonies. It will be seen from Table I that with few exceptions they fermented adonitol and sucrose. They varied in their dulcitol and inulin reactions and as a rule failed to liquefy gelatin within 3 months.

The thirty-four motile cultures of this subgroup consisted of twenty-four cultures of inositol-positive strains of MacConkey's type no. 102, and ten cultures of types which were very similar to *B. cloacae* types, but were inositol-positive. These motile cultures were chiefly responsible for the positive gelatin reactions shown in Table I. The cultures, which resembled *B. cloacae* types, were adonitol-negative, and taking into account the high correlation between the inositol and adonitol reactions of such Koser-positive types, the conclusion might well be drawn that these ten cultures were *B. cloacae* types giving atypical inositol reactions. To overcome the difficulty presented by such border-line cultures, both the inositol and adonitol reactions might be

[1] Differing from MacConkey's type in inositol reaction.

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used as subgroup criteria, subgroups 1, 2, 3 and 6 consisting of types which are negative either in inositol or adonitol, and subgroups 4, 5, 7 and 8 consisting of types giving positive reactions in both inositol and adonitol. The adoption of such a system would necessitate the regrouping of only twelve cultures in the table, namely, one culture in subgroup 7, and eleven cultures in subgroup 8; and it is doubtful if the extra trouble would be justified. Further, it is possible that a typical inositol-fermenter might give a negative (i.e. an atypical) adonitol reaction. This appears to be the case with the adonitol-negative culture of subgroup 7.

The view is widely held that *B. aerogenes* types are not normal inhabitants of the human and animal intestine and are of rare occurrence in faeces, but are common in the soil, on plants and grains and in water, or in other words, they are non-faecal organisms. On the other hand MacConkey (1909) isolated his strains of no. 103 most frequently from human faeces and in only a few cases from soil and cheese. Ford (1927) states that *B. lactis aerogenes* occurs chiefly in bovine faeces, while according to Sherman (1935), *B. aerogenes* types occur constantly in animal faeces, though in relatively small numbers. Cruickshank & Cruickshank (1931), Hay (1932) and Bardsley (1934) showed that such organisms were present in small numbers in most specimens of human faeces. Bardsley also found that they occurred less frequently than *B. coli* types in upland surface waters, in water from shallow wells and springs, and in soil. The writer obtained 233 cultures of this subgroup, 105 from milk and 128 from bovine faeces. With four exceptions the cultures from bovine faeces were obtained by enrichment methods, these cultures being isolated from approximately one-half of the number of specimens examined. It is therefore evident that these organisms are frequently present in small numbers in bovine faeces as in human faeces.

Anomalous strains. Thirty-nine cultures were atypical and were consequently not included in these subgroups. Twenty of these cultures apparently belonged to the Koser-positive subgroups, 3 (seven cultures), 6 (seven cultures), 7 (three cultures) and 8 (three cultures), but they did not produce sufficient growth in Koser's medium to give positive reactions. In all other respects they were similar to the other types of the respective subgroups. The remaining nineteen anomalous cultures appeared to be *B. cloacae* strains giving positive indole reactions. Such cultures are comparatively rare, but it is commonly accepted that they do occur. Thus Ruchhoft *et al.* (1931) state that it is well known that *B. cloacae* produces indole and they report that such atypical strains are occasionally present in water. They also note that Bahlman & Sohn's results indicate that these organisms are seldom found in routine work. Eleven of the indole-positive *B. cloacae* cultures isolated by the writer did not grow sufficiently in Koser's medium to produce positive Koser reactions. Such Koser-negative, indole-positive *B. cloacae* strains are of interest as they may be regarded as intermediate types, forming a link between the *B. coli* and *B. cloacae* subgroups.

A number of the thirty-nine anomalous cultures did not give well-defined reactions. Thus some of the indole-positive *B. cloacae* types appeared to produce only traces of indole. The colour change on the addition of the indole reagent, being so slight that confirmatory tests had to be made. Some of the Koser-negative atypical cultures produced very slight turbidity in Koser's citrate medium; it is possible that these cultures should have been recorded as Koser-positive. It is worthy of note that most of the anomalous cultures were isolated from milk.

Border-line strains, i.e. those which appear to belong to recognized types but are atypical with regard to one or other of the subgroup criteria, present a problem in the classification of coliform bacteria, no matter what criteria are used. However, by employing the Voges-Proskauer, Koser, inositol and indole reactions for the differentiation of subgroups, the number of such cultures obtained is comparatively small, amounting in this investigation to fifty-one cultures (including the twelve Koser-positive cultures which were inositol-positive and adonitol-negative) out of a total of 1636, i.e. 3·1%. There appears to be no reason why such anomalous cultures should not be included in the subgroups with which, apart from their atypical reactions, they have the most characters in common. In any case, in routine work it is as a rule sufficient to distinguish between typical *B. coli* and other coliform types, and as the former (subgroup 1) are particularly well-defined owing to the correlation which exists between the subgroup criteria (see later), the problem with regard to the classification of such border-line strains does not arise.

THE CORRELATION BETWEEN VARIOUS BIOLOGICAL CHARACTERS

In determining the correlation shown by a group of organisms with regard to particular characters, all possible combinations of these characters are considered. Thus if two characters x and y have either positive or negative values in the case of different types of bacteria belonging to a particular group, the frequency of all possible combinations of positive or negative values for these characters (e.g. $+x,+y$; $+x,-y$; $-x,+y$; $-x,-y$) is taken into account. The coefficient of association can be calculated by means of the formula $\frac{ad-bc}{ad+bc}$, where a equals the number of cultures of the various types with $+x,+y$ reactions; b, the number with $+x,-y$ reactions; c, the number with $-x,+y$ reactions; and d, the number with $-x,-y$ reactions (see Yule, 1937).

The Voges-Proskauer and methyl-red reactions. There was an almost complete correlation between the Voges-Proskauer and methyl-red reactions. Thus in the case of the 797 cultures obtained by the writer from milk, the coefficient of association was −0·999.

The Voges-Proskauer and Koser reactions. There was a high correlation between the Voges-Proskauer and Koser reactions, the coefficient of association being +0·980. Of 547 Voges-Proskauer-positive cultures, only twenty-four were Koser-negative, and all these Koser-negative cultures were atypical strains. On the other hand, of 1089 Voges-Proskauer-negative cultures, 889 were Koser-negative and 200 Koser-positive. Again, of 913 Koser-negative cultures, 889 were Voges-Proskauer-negative, but only 523 out of 723 Koser-positive cultures were Voges-Proskauer-positive. The Voges-Proskauer-negative Koser-negative cultures belonged to subgroup 1, the typical *B. coli*; the Voges-Proskauer-negative

Koser-positive cultures belonged to subgroups 2, 3, 4 and 5. A large proportion (159 out of 200) of these Voges-Proskauer-negative Koser-positive cultures were of indole-negative types. On the other hand, the Voges-Proskauer-negative Koser-negative cultures, with rare exceptions, were indole-positive.

The Voges-Proskauer and inositol[1] *reactions.* A high correlation was found between the Voges-Proskauer reaction and the fermentation of inositol, the coefficient of association being +0·910. Most of the Voges-Proskauer-negative cultures (773 out of 826), including all the cultures which were Voges-Proskauer-negative, Koser-negative and indole-positive, i.e. subgroup 1 (or typical *B. coli*), failed to ferment inositol. Voges-Proskauer-negative inositol positive types belonged to subgroups 4 and 5. As regards the Voges-Proskauer-positive cultures, 291 (chiefly of *B. aerogenes* and *B. oxytocus* types) gave positive inositol reactions and 199 (chiefly *B. cloacae* types) gave negative. Again, out of 344 inositol-positive cultures, 291 (84·6%) were Voges-Proskauer-positive, and of 972 inositol-negative cultures, 773 (79·5%) were Voges-Proskauer-negative.

The Voges-Proskauer and indole reactions. The coefficient of association between the Voges-Proskauer and indole reactions was −0·929. Of 1089 Voges-Proskauer-negative cultures, 923 were indole-positive, the exceptions being chiefly Koser-positive types (subgroups 3 and 5). Cultures giving negative Voges-Proskauer, Koser and indole reactions were seldom obtained. Of the 547 Voges-Proskauer-positive cultures, 454 were indole-negative (chiefly *B. aerogenes* and *B. cloacae* types) and 93 indole-positive (chiefly *B. oxytocus* types). Further, with regard to the indole-positive cultures, only 93 out of 1016 were Voges-Proskauer-positive and these were chiefly of subgroup 7, i.e. *B. oxytocus* types. As regards the 620 indole-negative cultures, 166 were Voges-Proskauer-negative and 454 Voges-Proskauer-positive.

The Koser and inositol reactions. The coefficient of association between the Koser and inositol reactions was high, being +0·987. Koser-negative strains were almost always inositol-negative, there being only five inositol-positive cultures out of 675 Koser-negative. On the other hand, out of 723 Koser-positive cultures, 363 were inositol-negative and 360 inositol-positive. The inositol-fermenters were almost always Koser-positive, only five out of 344 inositol-positive cultures (1·5%) being Koser-negative. The inositol-negative cultures, however, varied in their Koser reactions according to the indole reactions, the indole-positive types, e.g. *B. coli*, in most instances (659 out of 683, i.e. 96·5%) giving negative Koser reactions, and the indole-negative types, e.g. *B. cloacae*, in most cases (278 out of 289, i.e. 96·2%) giving positive Koser reactions. The twenty-four cultures which were inositol-negative, indole-positive and Koser-positive belonged chiefly to subgroup 2 and were in most instances Koser-positive strains of MacConkey's types nos. 5, 34 and 71. The eleven cultures which were inositol-negative, indole-negative and Koser-negative, were atypical strains, being chiefly Koser-negative cultures of MacConkey's types nos. 7, 73, 74, 108 and 109.

The Koser and indole reactions. There was an almost complete inverse correlation between the Koser and indole reactions, the chief exceptions being types which were both inositol- and indole-positive, e.g. those of subgroups 4 and 7. These inositol-positive indole-positive strains were as a rule Koser-positive, only two being Koser-negative out of ninety-five. While the coefficient of association between the Koser and indole reactions was −0·992, it would have been as high as −0·998, if these inositol-positive indole-positive cultures had been excluded. Accordingly, indole-negative cultures are Koser-positive, while indole-positive cultures are Koser-positive or negative according to whether the inositol reactions

[1] The inositol test was not used in the early stages of the investigation of the coliform flora of milk, and therefore the inositol reaction has been determined in only 1316 cultures. Consequently, in stating the correlation between the inositol reaction and other characters, the figures given do not correspond with those of Table I.

are positive or negative. There are very few exceptions to this rule. This correlation between the indole and Koser reactions holds good irrespective of the nature of the Voges-Proskauer reaction.

The inositol and indole reactions. The coefficient of association between the inositol and indole reactions was low, being only −0·722. However, cultures which were both indole-positive and Koser-negative were almost always (659 out of 661) inositol-negative.

The inositol and adonitol reactions. There was an almost complete correlation between the inositol and the adonitol reactions with the exception of adonitol-positive types of subgroup 1, e.g. MacConkey's types nos. 1, 2, 33 and 100. These *B. coli* strains, like all other strains which were both Koser-negative and Voges-Proskauer-negative, gave in all instances negative inositol reactions. If these Koser-negative Voges-Proskauer-negative strains had been excluded, the coefficient of association between the inositol and adonitol reactions would have been +0·997. It is noteworthy that while the inositol reactions of certain types showed this correlation with adonitol, they differed from the inositol reactions noted by MacConkey for types which were similar to them in all their other characters. Thus these cultures which appeared to be of MacConkey's types nos. 66 and 97 were found to be inositol-positive instead of negative, and those of type no. 75 were found to be negative instead of positive. Further, the cultures which were apparently of type no. 104 gave positive inositol reactions, and those of type no. 109 gave negative reactions, whereas according to MacConkey, they both produce acid only in inositol.

The inositol and other reactions and motility. There was a much higher correlation between the inositol reaction and motility than between the Voges-Proskauer, Koser and indole reactions and motility. The coefficient of association in the 1316 cultures tested in inositol was −0·967. Thus of 972 non-inositol-fermenting cultures, only 104 (10·7%) were non-motile. These non-motile cultures belonged chiefly to subgroup 1, being non-motile *B. coli* types, e.g. MacConkey's types nos. 2, 5, 35, 72 and 107. As regards the 344 inositol-fermenting cultures, only forty-two (12·2%) were motile; these motile inositol-fermenting cultures belonged chiefly to MacConkey's type no. 102 (Voges-Proskauer-positive, Koser-positive, inositol-positive, indole-negative, gelatin-positive and motile) and to a type which resembled no. 108 (*B. cloacae*) but gave positive inositol reactions. (The absence of motility in inositol-fermenters can be accounted for by the presence of encapsulation.)

Voges-Proskauer-negative cultures were most frequently (934 out of 1089) motile, but only 259 out of the 547 Voges-Proskauer-positive types were motile.

Koser-negative cultures were as a rule motile, only 105 out of 913 being non-motile. These non-motile cultures were chiefly non-motile *B. coli*, e.g. MacConkey's types nos. 2, 35, 72 and 107. On the other hand, as regards the 723 Koser-positive cultures, 385 were motile (e.g. *B. cloacae* types) and 338 were non-motile (e.g. *B. aerogenes* and *B. oxytocus* types).

The majority of the indole-positive cultures were motile (820 out of 1016), but only 373 of the 620 indole-negative cultures were motile.

Other biochemical reactions. The sucrose, raffinose, dulcitol and inulin reactions did not show a high degree of correlation with the Voges-Proskauer, Koser, inositol and indole reactions (see Table I). However, cultures of subgroups 4, 5, 6, 7 and 8 (i.e. all the inositol-positive subgroups and also the *B. cloacae*), with the exception of only one culture, fermented sucrose; and all the cultures of subgroups 1, 2 and 3 failed to ferment inulin. The raffinose reaction correlated almost completely with the sucrose, the coefficient of association in the 797 cultures obtained from milk being +0·9998. The Koser-negative cultures rarely liquefied gelatin, only nine out of 913 liquefying gelatin within three months. But 231 of the 723 Koser-positive cultures were gelatin-liquefiers. All the cultures fermented mannitol. As regards the action on salicin, Koser-positive types, i.e. types of subgroups 2–8, in most instances (560 out of 583) gave positive reactions, while Koser-negative types, i.e. types of subgroup 1, varied in their salicin reactions but were most frequently (383 out of 465)

positive.[1] It was also found that different strains of even classic types of coliform bacteria varied in their salicin reactions. Thus sixty-eight of the cultures of *B. coli communis* (Escherich) isolated from milk gave positive salicin reactions and twenty-six gave negative; 181 of the cultures of *B. coli communior* from the same source gave positive reactions and twelve gave negative; thirteen of the cultures of MacConkey's type no. 4 gave positive reactions and thirteen gave negative. Mackie (1921) obtained similar results with cultures from human faeces and other sources. The writer found that with the majority of the types in which salicin-negative cultures could be obtained, there was a preponderance of cultures giving positive reactions.

The value of various characters for systematic purposes

One of the chief difficulties in the classification of coliform organisms arises from the fact that they show great variation with regard to certain characters, the true value of which for differential purposes is not known. In this connexion it must be borne in mind that with bacteria in general it is frequently difficult to assess the value of characters for systematic purposes. Certain features, owing to their morphological or biological importance and also to their stability, are universally recognized to be valuable and reliable criteria for differentiating types and even groups, e.g. shape of the cell, spore formation, position of flagella on the cell, reaction to Gram's stain, acid-fastness, ability to produce specific disease, ratio of CO_2 to H_2 in the fermentation products, etc. Other characters, which appear to be of minor morphological or biological importance, but which are constant features of the organisms, are also used, e.g. fermentation of sugars, liquefaction of gelatin, etc. A greater importance can be attached to the latter if they show some relationship to other features of the organism. The value of any character for systematic purposes depends therefore not only on its morphological or biological importance, but also on the degree to which it is correlated with other characters.

The Voges-Proskauer reaction. The importance of the Voges-Proskauer reaction for differential purposes has been stressed by Levine (1916*a*, *b*, *c*, 1918, 1921), Johnson (1916), Hulton (1916), Johnson & Levine (1917), Rogers *et al.* (1918), Chen & Rettger (1920), Bergey (1923, 1934), Ruchhoft *et al.* (1931) and many others. Its great value as a criterion in systematic work is indicated by the fact that it shows an almost complete correlation with the acid-producing power (or methyl-red reaction), with the amount of gas produced and with the ratio of CO_2 to H_2 in the fermentation products. Differences in these features are indices of marked differences in the metabolism of the organisms. The Voges-Proskauer-negative types, subgroups 1, 2, 3, 4 and 5 (the genus *Escherichia* of Bergey), produce in carbohydrate media a high acidity (a methyl-red-positive reaction), a moderate amount of gas and a low gas ratio (the CO_2 to H_2 ratio ranging from 1 to 0·5). On the other hand, the Voges-Proskauer-positive types, subgroups 6, 7 and 8 (the genus *Aero-*

[1] The salicin reaction was determined in the case of only 1048 cultures.

bacter of Bergey), produce a low acidity (a methyl-red-negative reaction), a comparatively large amount of gas and a high gas ratio (the CO_2 to H_2 ratio ranging from 2 to 3). Further, Voges-Proskauer-positive types are almost always Koser-positive. Voges-Proskauer-negative types are Koser-negative in the case of subgroup 1 (*B. coli* types), and Koser-positive in the case of subgroups 2, 3, 4 and 5. In Bergey's classification (1934), the Voges-Proskauer reaction is employed as the sole criterion for the primary subdivision of the coliform group into two genera, and as a result these Voges-Proskauer-negative, Koser-positive types (subgroups 2, 3, 4 and 5) are placed in the same genus, *Escherichia*, as the Voges-Proskauer-negative, Koser-negative types (subgroup 1 or *B. coli* types). However, types of subgroups 4 and 5 in many respects resemble very closely subgroup 8, the *B. aerogenes* types. Thus they are not only Koser-positive (and in the case of subgroup 5, the *B. friedländeri* types, indole-negative) but they also have very high fermentative powers, fermenting in all instances inositol, adonitol and sucrose. Moreover, like *B. aerogenes*, they are non-motile, with relatively high powers of resistance to brilliant green and with the ability to produce large thick mucoid colonies and become encapsulated. Accordingly, these types are intermediate in their characters between the *B. coli* and the *B. aerogenes*. On the other hand, subgroup 6 (*B. cloacae*) being Voges-Proskauer-positive, methyl-red-negative and of high gas ratio, is included by Bergey in the genus *Aerobacter*. Nevertheless, the intermediate character of these types is shown by the fact that, like *B. coli*, they do not ferment inositol and with rare exceptions, adonitol, and are as a rule motile.

The Koser reaction. The value of the Koser reaction for the grouping of coliform types has been shown by Raghavachari (1926), Hicks (1927), Perry (1929), Ruchhoft *et al.* (1931), Hay (1932), the writer (1933, 1935) and Bardsley (1934). Its employment for systematic purposes enables subgroup 1 or *B. coli* (Koser-negative types) to be differentiated from the other subgroups (Koser-positive types). With very few exceptions, Koser-negative coliform types are Voges-Proskauer-negative, inositol-negative and indole-positive, or in other words, are typical *B. coli*. Further there is an almost complete negative correlation between the Koser and indole reactions with the exception of indole-positive, inositol-fermenting types (subgroups 4 and 7)—these as a rule are Koser-positive. The Koser reaction is also an important index of the viability of different types of coliform organisms under artificial and natural conditions. Thus Koser-positive cultures (inositol-negative strains as well as inositol-positive) are more highly resistant to brilliant green than Koser-negative (Malcolm, 1935). The greater vigour of the Koser-positive strains, as compared with the Koser-negative, is also shown by their ability to attack the citrate radicle; their greater viability in polluted streams and in stored water (Clemesha, 1912; Rogers, 1918; Winslow & Cohen, 1918; Platt, 1935); their ability in many instances to ferment inositol, and by the fact that they can frequently become encapsulated.

The inositol reaction. The importance of the inositol reaction for the classification of coliform bacteria has been shown by Mackie (1921), Hay (1932) and the writer (1935). In the case of Voges-Proskauer-negative types, it sharply differentiates subgroups 1 (*B. coli*), 2 and 3, from subgroups 4 and 5 (*B. friedländeri*); and in the case of Voges-Proskauer-positive types, it differentiates subgroup 6 (*B. cloacae*) from subgroups 7 (*B. oxytocus*) and 8 (*B. aerogenes*). Its employment as a group criterion is justified by the well-marked features of the inositol-fermenting types (subgroups 4, 5, 7 and 8) which may be recapitulated briefly as follows: encapsulation with consequent production of large, mucoid colonies, and as a rule the absence of motility; very wide fermentative activity—with few exceptions, adonitol- and sucrose-positive; a positive Koser reaction; the possession of relatively high powers of resistance to brilliant green; in most instances a positive Voges-Proskauer reaction. On the other hand, the non-inositol-fermenters (subgroups 1, 2, 3 and 6) are as a rule motile and have not so high fermentative powers as the inositol-fermenters. Also, except for subgroup 2, they vary in their Koser reactions according to their indole reactions, the indole-negative types giving positive Koser reactions and vice versa. Without exception, types which are Koser-negative, indole-positive and Voges-Proskauer-negative, i.e. subgroup 1 or *B. coli* types fail to ferment inositol. This rule applies even to the types of this subgroup, which ferment adonitol; these are the only coliform types failing to show a positive correlation between the inositol and adonitol reactions. Further, the Koser-negative, non-inositol-fermenters (i.e. subgroup 1) have comparatively low powers of resistance to brilliant green, do not become encapsulated and fail to produce large, mucoid colonies; the Koser-positive non-inositol-fermenters have as a rule comparatively high powers of resistance to brilliant green, and some of the types belonging to subgroup 3, and many belonging to subgroup 6, become encapsulated and produce large, thick mucoid colonies.

The indole reaction. Mackie (1921), Hicks (1927), Perry (1929), Ruchhoft *et al.* (1931), the writer (1933, 1935) and others have stressed the importance of the indole reaction for the differentiation of coliform types and subgroups. Its employment, along with the other three reactions, for systematic purposes enables subgroup 1, the *B. coli*, to be very well-defined—a most important matter in public health work. Further, it enables subgroup 2 to be distinguished from subgroup 3; and subgroup 4 from subgroup 5. It also allows subgroup 7 (*B. oxytocus*) to be differentiated from subgroup 8 (*B. aerogenes*) and as a result the action on gelatin does not require to be determined. (*B. oxytocus* types differ chiefly from *B. aerogenes* in that they produce indole and slowly liquefy gelatin.)

Indole-negative types are Koser-positive; while indole-positive types are Koser-negative or positive according to whether the inositol reactions are negative or positive respectively. There are very few exceptions to this rule and these belong chiefly to subgroup 2. Thus the *B. cloacae* types (subgroup 6)

and *B. aerogenes* types (subgroup 8) are indole-negative and Koser-positive; the typical *B. coli* (subgroup 1) are indole-positive, Koser-negative and inositol-negative; and the *B. oxytocus* types (subgroup 7) are indole-positive, Koser-positive and inositol-positive. Moreover, there is a high correlation between the indole and Voges-Proskauer reactions.

Other biochemical reactions. The sucrose, raffinose, dulcitol and inulin reactions have not been employed for the differentiation of subgroups, because these reactions do not correlate highly with the Voges-Proskauer, Koser, inositol and indole reactions. It is, however, worthy of note that subgroups 4, 5, 7 and 8, i.e. all the inositol-positive subgroups and also subgroup 6, the *B. cloacae*, are sucrose-positive, while subgroups 1, 2 and 3 are inulin-negative. The adonitol reaction correlates almost completely with the inositol with the exception of adonitol-positive types of subgroup 1 (the *B. coli* subgroup). These types are similar in other characters to other types of this subgroup, so that there appears to be no necessity to place them in a separate group. Consequently, the adonitol reaction has not been used as a group criterion. However, it may be substituted for the inositol reaction to reduce expenses in routine work, provided it is borne in mind that Koser-negative Voges-Proskauer-negative types, e.g. the *B. coli* subgroup, are inositol-negative, even although they give positive adonitol reactions. The mannitol reaction is of no value for differentiating subgroups or types as all lactose-fermenting coliform bacteria ferment this substance. The salicin reaction is also of no value as a subgroup criterion, as types of subgroups 2–8 (the Koser-positive types) as a rule give positive reactions while types of subgroup 1 vary greatly in their salicin reactions, but are most frequently positive. As has already been shown, different cultures of even classic types of coliform bacteria vary in their salicin reactions.

While the gelatin reaction is of value for the differentiation of rapid gelatin-liquefying types of bacteria, e.g. *Proteus*, from slow liquefying or non-liquefying types, e.g. *B. coli*, it is of little value for the differentiation of subgroups or types of coliform bacteria. Those coliform cultures which liquefy gelatin do so in most instances very slowly, the process taking frequently from 4 to 12 weeks at 20 or 37° C. A fairly high proportion (approximately one-third in the writer's investigations) of the cultures of classic gelatin-liquefying types, e.g. MacConkey's types nos. 108 (*B. cloacae*) and 65 (*B. oxytocus perniciosus*), may fail to liquefy gelatin within 3 months. Gelatin-liquefying types of coliform bacteria may be differentiated as a rule from non-liquefying types by other characters. Thus liquefying types are rarely Koser-negative. They are generally Koser-positive, Voges-Proskauer-positive types which are either motile, e.g. *B. cloacae*, or non-motile and indole-positive, e.g. *B. oxytocus*. (MacConkey's types nos. 36 and 70 are liquefying but are not included in either of these categories. They are Koser-positive, motile, indole-negative and Voges-Proskauer-negative. They resemble in these characters certain non-liquefying types, e.g. MacConkey's types nos. 7, 74 and 109, but they can be distinguished

from the latter by other biochemical reactions, and anyway, they are of comparatively rare occurrence.)

Motility. As the coefficient of association between motility and the inositol reaction is high (−0·967), the inositol-negative subgroups are characterized by motility, and the inositol-positive by non-motility. The chief exceptions to this rule are certain non-motile inositol-negative types which are commonly accepted as being typical *B. coli* (subgroup 1), e.g. MacConkey's types nos. 2, 5, 35, 72 and 107. There appears to be no need to group these apart from the motile *B. coli* types. The few inositol-fermenting cultures, which are motile, belong chiefly to MacConkey's type no. 102 or are of atypical strains. Cultures of no. 102 are similar to *B. aerogenes* types in their Voges-Proskauer, Koser, inositol and indole reactions and are therefore included in that subgroup. Accordingly, it would appear that if the inositol reaction is employed as a group criterion, there is no need to use in addition the presence or absence of motility. Bergey (1923, 1934) subdivides the Voges-Proskauer-positive types, the genus *Aerobacter*, according to motility and thus differentiates *B. cloacae* types from *B. aerogenes* and *B. oxytocus*. On the other hand, by employing the Voges-Proskauer, Koser, inositol and indole reactions as group criteria, the *B. cloacae* types are differentiated from the *B. aerogenes* and *B. oxytocus* types by the inositol reaction, and the *B. oxytocus* from the *B. aerogenes* by the indole reaction.

Discussion

(1) The members of the coliform group are apparently so numerous and so closely interlinked, that no matter which feature is used for differentiating purposes, types can generally be distinguished which are identical in all other respects. Thus, although the Voges-Proskauer-negative types (the *Escherichia* types of Bergey (1923, 1934)) differ from the Voges-Proskauer-positive (the *Aerobacter* types of Bergey) in their manner of carrying out fermentation, nevertheless, with regard to other characters, the former frequently present identical features to the latter, as in *B. friedländeri* (a Voges-Proskauer-negative or *Escherichia* type) and *B. aerogenes* (a Voges-Proskauer-positive or *Aerobacter* type). Again, if the salicin reaction is used as a differential in addition to those characters employed by MacConkey, it is possible to distinguish two types of each of such classic organisms as *B. coli communis* Escherich (*Escherichia communis*) and *B. coli communior* (*Escherichia communior*). In a few instances a character shows a very high correlation with one or more other characters, but even in such cases, types can generally be found in which the correlation does not occur. Even with the classic types of coliform bacteria, individual cultures may be found which have atypical features. Thus cultures may be similar in their characters to *B. lactis aerogenes* (*Aerobacter aerogenes*) or to *B. cloacae* (*Aerobacter cloacae*) except that they fail to give Koser-positive reactions.

Further, the environmental origin of different types is now considered to

be much less significant since it has been shown that the so-called non-faecal types (the *aerogenes-cloacae* types) are almost always present in human and animal faeces, although in relatively small numbers under normal conditions. While these organisms may be present in soil and water and on fodder and grains, even in absence of recent faecal contamination, it would appear from the findings of Bardsley (1934) and others that, like the *B. coli* types, they are not widely distributed in nature except where there has been at some time or other faecal contamination.

Taking into account all these facts, it would appear undesirable to make an arbitrary division of the coliform group into two or more genera. The numerous types are so closely interlinked in characters and in relation to environment as to justify their inclusion in one genus. However, to facilitate the identification of types the group may be divided into eight subgroups, by employing the Voges-Proskauer, Koser, inositol and indole reactions as subgroup criteria. Of these subgroups, no. 1, i.e. the *B. coli* types or typical *B. coli* (Voges-Proskauer-negative, Koser-negative, inositol-negative, indole-positive) and no. 8, i.e. the *B. aerogenes* types (Voges-Proskauer-positive, Koser-positive, inositol-positive, indole-negative), present the most striking contrast with regard to such features as motility, encapsulation, amount of acid produced in carbohydrate media (methyl-red reaction), CO_2 to H_2 ratio, Voges-Proskauer reaction, ability to obtain their carbon from citrates and their nitrogen from uric acid, powers of fermentation, indole formation and resistance to brilliant green. The types comprising the other subgroups are intermediate in their characters between the *B. coli* and the *B. aerogenes*; thus the non-inositol-fermenting Voges-Proskauer-negative types, i.e. subgroups 2 and 3, resemble fairly closely the *B. coli*; the non-inositol-fermenting Voges-Proskauer-positive types, i.e. subgroup 6, or *B. cloacae* types, resemble in some respects the *B. coli* types and in other respects, the *B. aerogenes*; and the inositol-fermenters (Voges-Proskauer-negative as well as Voges-Proskauer-positive types), i.e. subgroups 4, 5 and 7, are very similar to the *B. aerogenes*.

(2) Of all the coliform subgroups, no. 1 (the *B. coli* or typical *B. coli*) is widely recognized to be of special importance. This is due to the fact that it includes those types which are by far the most prevalent in human and animal faeces, comprising the great majority of coliform cultures obtained from such sources when isolations are made by the usual methods (see Table II). Organisms of these types may also be found in soil, in water and on plants; but in absence of recent faecal contamination their incidence in such environment is comparatively low and in many instances they are not to be found. It would therefore appear that they thrive best in the intestines and cannot persist indefinitely in soil and water. On the other hand, all the other coliform subgroups, e.g. the *B. aerogenes*, *B. oxytocus* and *B. cloacae*, consist of types which occur in relatively small numbers in human and animal faeces, although they are almost always present, as can be demonstrated by enrichment methods. As already mentioned, they may be found in nature, e.g. in soil

and water and on grains, even in absence of recent faecal contamination, although they are apparently not widely distributed except where there has been, at some time or other, such contamination. However, they are generally the dominant coliform types in soil, being apparently better adapted than the *B. coli* types to live in a natural environment, but less adapted to live in the intestines. This is borne out by the following facts:

(*a*) The *B. aerogenes* types occur in fresh faeces as a rule only in small numbers; but as shown by Clemesha (1912), if water is polluted with sewage, these types, though rare at first, are extremely common after a few days. Rogers (1918) showed that they were able to survive longer than *B. coli* types in water held in bottles, and in running water and polluted streams; and his results as regards stored water are supported by the findings of Winslow & Cohen (1918) and Platt (1935).

(*b*) The inositol-positive types and the *B. cloacae* (subgroups 4–8) are frequently encapsulated, and therefore might be expected to be more resistant to adverse conditions generally than the non-capsulated coliform types which are prevalent in the intestines. Thus they can resist a higher concentration of brilliant green than the latter. In addition they can attack a much wider range of substances than the *B. coli* types.

Having regard to the foregoing, it is evident that the incidence of any type of coliform organism in soil or in water serves as an indication of faecal contamination. The presence of large numbers of subgroup 1 (*B. coli* types) in water is evidence of recent sewage pollution. On the other hand, if organisms of only subgroups 2–8, e.g. *B. aerogenes* and *B. cloacae* types, are present, there still remains the probability that faecal contamination has occurred, but not at a recent date.

The occurrence of large numbers of coliform organisms in milk is accepted as an index either of excessive contamination or of storage at too high temperatures. While the sources of these organisms may be various, e.g. the utensils, animal's skin, byre dust, etc., the fact must be borne in mind that bovine faeces contain large numbers of the *B. coli* types and almost always, in addition, the *B. cloacae*, *B. aerogenes* and other types (subgroups 2–8), though in smaller numbers. Consequently, there is always a possibility that bovine faeces may be the original source of such contaminants in milk, no matter whether they are of *B. coli* or other coliform types.

Summary

A study of the biological characters of 1636 cultures of coliform bacteria, isolated from milk and bovine faeces, shows that the coliform group consists of a large number of different types. These types are so closely interlinked in characters and in relations to environment as to justify their inclusion in one genus. Nevertheless, to facilitate the identification of types, the group may be subdivided into subgroups, the Voges-Proskauer, Koser, inositol and indole

reactions being reliable and outstanding criteria for this purpose. These characters show almost perfect correlations with various other characters. Thus Voges-Proskauer-negative types have a low CO_2 to H_2 ratio and are methyl-red-positive; Voges-Proskauer-positive types have a high CO_2 to H_2 ratio, are methyl-red-negative and Koser-positive. Koser-negative types are not highly resistant to brilliant green, are non-capsulated, do not form thick mucoid colonies and are Voges-Proskauer-negative, inositol-negative and indole-positive. Koser-positive types are highly resistant to brilliant green, and with the exception of group 2 and certain members of group 3, are frequently encapsulated and form thick, mucoid colonies. Non-inositol-fermenters are as a rule motile and adonitol-negative. Inositol-fermenters are Koser-positive, adonitol-positive, sucrose-positive and raffinose-positive; are frequently encapsulated and form thick, mucoid colonies; and as a rule are non-motile. Indole-negative types are Koser-positive, while indole-positive types are generally Koser-positive or negative according to whether the inositol reactions are positive or negative.

By means of these four characters (the Voges-Proskauer, Koser, inositol and indole reactions) it is possible to arrange coliform types in a series of well-defined subgroups (Table III).

Table III

Subgroup of types	Voges-Proskauer	Koser	Inositol	Indole	MacConkey's types	Habitat
1. *B. coli*	−	−	−	+	1, 2, 4, 5, 33, 34, 35, 71, 72, 100, 106, 107	Most prevalent coliform bacteria in intestines and faeces; less common in soil
2. Intermediate	−	+	−	+	As above but Koser-positive	Found occasionally in soil and faeces
3. Intermediate	−	+	−	−	7, 36, 70, 74, 109	Found frequently in soil and occasionally in faeces
4. Intermediate	−	+	+	+	66,[1] 101	Seldom found either in soil or faeces
5. *B. friedländeri*	−	+	+	−	68, 99, 104[1]	Found in upper respiratory tract and to a limited extent in the intestines and faeces
6. *B. cloacae*[2]	+	+	−	−	3, 69, 73, 75,[1] 105, 108	Found frequently in the intestines, faeces and soil, and on plants
7. *B. oxytocus*[2]	+	+	+	+	65, 97[1]	Found occasionally in faeces
8. *B. aerogenes*	+	+	+	−	67, 98, 102, 103	Found frequently in the intestines, faeces and soil, and on plants

[1] Differing from MacConkey's type in inositol reaction.

[2] Frequently liquefy gelatin.

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