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THE CLASSIFICATION of ORGANISMS belonging to the GROUPS, ESCHERICHIA and AEROBACTER, based upon the BIOLOGY of the TYPES met with in MILK and FAECES of CATTLE: with OBSERVATIONS on the FREQUENCY of the TYPES of COLIFORM ORGANISMS occurring in BOVINE FAECES and on the FACTORS which are responsible for the ENRICHMENT of AEROGENES-CLOACAE FORMS in MILK.

by

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This investigation is a continuation of that entitled "The Bacterial Contaminants of Milk and the Value of the various Methods for determining their Presence", for which the Degree of Ph.D. was awarded in 1933. It represents entirely new work, except for minor parts of Section IV.

C O N T E N T S

F O R E W O R D

Section I. of the thesis is a general review of the work of other investigators on the subject, and an outline of the present research; Sections II, III and IV, give detailed accounts of the investigation and the findings. Certain parts of the thesis have already been published under the titles "The types of Coliform bacteria in bovine faeces" (Journal of Dairy Research, Vol. 6, No.3, September, 1935) and "The classification of coliform bacteria" (Journal of Hygiene, Cambridge, Vol. 38, No.4, 10th August, 1938). The part entitled "The enrichment of aerogenes-cloacae types in milk held at low temperatures: with observations on the relative rates of growth of aerogenes-cloacae and B. coli types in milk at different temperatures", is in course of publication.

S E C T I O N I.

GENERAL REVIEW

The coliform bacteria comprise a large and important group of organisms which normally live as saprophytes but under certain conditions are pathogenic in character. Their existence was first demonstrated in 1885 by Escherich; he isolated from the intestines of infants a bacterium which he termed B. coli communis. This organism was found to be a normal commensal in the intestines of man and animals. The fact that it was very similar in its morphological and biological characters to the typhoid bacillus, first described by Eberth in 1880-81 and isolated by Gaffky in 1884, necessitated the development of methods whereby the two organisms - the one a normal commensal, the other the cause of an acute infection - could be differentiated from one another. In the years which followed, this led to a considerable

amount of research work whereby much information was obtained with regard to the biological characters of these organisms and their ability to grow in various selective media. At the same time other organisms were found to occur in the intestines and the faeces in the case of various intestinal infections. Thus the B. suipestifer was shown to be associated with swine fever, by Salmon and Smit in 1886; B. enteritidis with a form of "food poisoning", by Gaertner in 1888; B. gallinarum with fowl typhoid, by Kleir in 1889; B. paratyphosus A and B with paratyphoid fever, by Achard and Bensaude in 1896, Gwyn in 1898 and Schottmüller in 1900; B. dysenteriae Shiga and B. dysenteriae Flexner with dysentery, by Shiga in 1898 and Flexner in 1900. Moreover the coliform commensals occurring in the bowels were found to be of more than one type. Escherich not only discovered B. coli communis but also B. (lactis) aerogenes. He noted certain differences between these organisms. Jordan in 1890 isolated B. cloacae from sewage. Kruse (1894), Dyar and Keith (1894), Smith (1895), Refik (1896), Gordon (1897), Grimbert and Legros (1900), Durham (1900-1901), Ford (1901), Houston (1902-1903, 1905), Jordan (1903 Savage (1906) and others, selecting a number of differential features, recognised various types of coliform bacteria. In Durham's scheme of classification, lactose-

fermenting organisms were divided into three groups, the prototypes of which were B. coli communis, B. coli communior and B. (lactis) aerogenes. Durham noted that B. friedländeri was closely related to B. (lactis) aerogenes, and included it in the same group. By the use of a much larger number of differential criteria MacConkey (1905, 1909), Bergey and Deehan (1908), Jackson (1911) and others were able to make more comprehensive classifications, and show that the coliform group consisted of a very large series of closely related types.

In conjunction with this systematic study of coliform bacteria, investigations were being made to determine the incidence of the organisms in the human and animal body and elsewhere. The fact was soon established that they are normal commensals in the human and animal intestine and are practically always present in faeces. They were also found by Kruse (1894), MacConkey (1905, 1909) and others to occur to a limited extent in soil and water and on vegetation and cereal grains, the numbers present in natural environments being much greater where there had been recent contamination with sewage or animal excrements. The importance of these findings in relation to the examination of water supplies for purity was early recognised by Public Health Authorities. The fact that

the causative organisms of a number of important infectious diseases may occur in the faeces of individuals suffering from these diseases or of "carriers", and that such organisms may persist for a considerable time in a natural environment, showed that water contaminated with sewage may be responsible for the spread of these infections. If such water is used for drinking purposes, it is at all times a potential source of infection. Accordingly in the bacteriological examination of water supplies a test was required which would indicate not necessarily the actual presence of the pathogens but the occurrence of faecal contamination. As coliform bacteria had been found to occur in practically all specimens of faeces but only to a limited extent in natural waters relatively free from faecal pollution, the absence of such organisms from given quantities of the water was recognised to be a valuable index of its purity, and consequently the "coliform test" was introduced in water analysis (Smith 1893 a,b, and 1895; Klein and Houston 1897-1898; Clark 1898, 1899; Houston 1899-1900, 1900-1901, 1901-1902; Fuller 1899; Irons 1900, 1902; Pakes 1900; Chick and Boyce 1900, 1901; Jordan 1901; Horrocks 1901, 1903; Makgill 1901; Savage 1901, 1902, 1906).

When as a result of the systematic work of the earlier investigators, it was recognised that there were

many different types of coliform bacteria, the environmental origin of the various types formed the subject of investigations by numerous workers, of whom the following are the most important: MacConkey (1905, 1906 and 1909), Winslow and Walker (1907), Ferriera, Horta and Paredes (1908), Clemesha (1912), Rogers, Clark and Evans (1914, 1915 and 1916), Levine (1916 a, b, c, d, e, 1918, 1921), Greenfield (1916), Hulton (1916), Johnson (1916), Rogers (1916 and 1918), Murray (1916), Burton and Rettger (1917), Hunter (1917), Johnson and Levine (1917), Rogers, Clark and Lubs (1918), Winslow and Cohen (1918), Wood (1919-1920) Winslow, Kligler and Rothberg (1919), Chen and Rettger (1920), Mackie (1921), Perry and Montfort (1921), Redman (1922), Koser (1924), Levine and Linton (1924), Bardsley (1926 and 1934), Jordan (1926), Tonney and Noble (1930), Ruchhoft, Kallas, Chinn and Coulter (1931), Kon (1933), Burke-Gaffney (1932, 1933) and others. The results of these investigations showed that the B. coli, otherwise called "typical" B. coli (see Muir and Ritchie, 1937), are the prevalent types in human and animal faeces, but in absence of recent faecal contamination they do not occur to any extent in water and soil and on vegetation and cereal grains. The other coliform types, e.g., B. aerogenes, B. cloacae, etc., (hereafter referred to as aerogenes-

cloacae) were in most instances found to be seldom present in faeces but frequently in the soil and water and on vegetation, ensilage and cereal grains. The fact that such bacteria could be found in soils and in water supplies where it was claimed there was little or no possibility of faecal contamination, gave rise to the widely accepted view that these organisms are not normal commensals in the intestine but are saprophytes occurring naturally in soil and water and on vegetation and that therefore their presence in a water supply does not necessarily indicate sewage pollution. Consequently, doubts were raised as to the value of the coliform test in the bacteriological analysis of water, the view being held that water supplies giving positive coliform tests were not necessarily faecal contaminated unless the typical B. coli were found to be present. Thus with specimens giving positive coliform tests the practice was adopted of determining the various types of coliform organisms present.

On the other hand, according to the results of certain investigators aerogenes-cloacae types are commonly present in faeces. Thus MacConkey (1905 and 1909) isolated his strains of no.65 (B. oxytocus) and no.103 (B.(lactis) aerogenes) most frequently from human faeces and in only a few cases from soil and cheese. He failed

to find any in animal faeces and on grains. Rogers, Clark and Evans (1914) rarely found aerogenes types in bovine faeces, but Rogers, Clark and Lubs (1918) frequently obtained them in human faeces. Of 177 strains, which they isolated from human faeces after preliminary enrichment in milk at 20°C., forty-six were aerogenes types. Moreover Cruickshank and Cruickshank (1931) and Hay (1932) showed by enrichment methods that aerogenes-cloacae types were present in small numbers in most specimens of normal human faeces. Thus it would appear from the findings of these workers that such organisms are of common occurrence as commensals in the intestine and that their presence in water supplies may well be due to faecal contamination. They are, however, more resistant in natural waters than the B. coli types (Clemesha 1912, Rogers 1918, Winslow and Cohen 1918, Gray 1932, Burke-Gaffney 1933, Platt 1935,) and their presence in such water in absence of typical B. coli, signifies that faecal contamination has probably occurred, either directly or indirectly, but not at a recent date. On the other hand, the presence of large numbers of the typical B. coli in water is evidence of recent sewage pollution.

The employment of the coliform test in water analysis was largely responsible for its adoption as an

indicator of excessive contamination of milk. The fact that various important infectious diseases may be spread by milk was early recognised by public health workers. It was also realised that if care is taken in the production and treatment of milk, there is less risk of its being contaminated not only with pathogenic organisms, but also with the bacteria occurring in bovine faeces, byre dust, etc. Park (1901), von Freudenreich (1902, 1903 and 1904), Burr (1902), von Freudenreich and Thoni (1903), Lux (1904), Willem and Miele (1905) and others, had found that milk drawn under clean conditions directly from the udder into sterile bottles contained comparatively few bacteria. Von Freudenreich (1902, 1903 and 1904), Burr (1902), Conn (1902), von Freudenreich and Thoni (1903), Harrison (1905), Heinemann (1905), Houston (1905), MacConkey (1906) and others, showed that under such conditions colifor bacteria are generally absent from milk, but that they frequently gain access to it from extraneous sources after it i drawn, especially if there has been excessive contamination. Various workers - Park (1901), Nicolle and Petit (1903), Conn and Esten (1904), Koning (1905), Harrison (1905), Houston (1905), etc., - had also found that there was little or no multiplication of bacteria, including coli-

form types, in milk which is rapidly cooled and held at temperatures below 10 or 11°C. Accordingly the incidence of coliform organisms, as determined by the coliform test, in milk which had been rapidly cooled and held at low temperatures, was widely accepted as an index of the extent of its contamination with extraneous bacteria, especially those from faecal sources, and consequently where doubts arose as to the "purity" of the milk, i.e., freedom from excessive contamination or from taints, the coliform test was very frequently carried out. Houston (1905) recognised the great value of this test, along with certain other tests, for the grading of cooled milk according to its relative freedom from contamination. He suggested certain standards for his nine different "classes of milk" - no coliform bacteria in 100 c.c. (First Class); none in 10 c.c. (Second Class); none in 1 c.c. (Third Class), etc. In 1923 the coliform test was adopted by the Ministry of Health for England and the Department of Health for Scotland as one of the tests for the grading of milk. Thus according to the Milk (Special Designations) Order, 1923, milk of the "Certified" grade must not contain coliform bacteria in 1/10 ml., and milk of "Grade A (Tuberculin Tested)" and "Grade A" standards must not contain coliform bacteria in 1/100 ml. These

standards have been retained for the corresponding grades of milk under the terms of the Milk (Special Designations) Order, 1936.

The coliform test, as carried out for statutory purposes by Public Health Authorities, consists merely in the detection of the presence or absence of coliform organisms in certain quantities of the milk (i.e. the approximate determination of their incidence in the milk) and therefore no information is obtained with regard to the types present. This is regarded as a serious disadvantage by certain workers who are of the opinion that the aerogenes-cloacae types which occur in milk, are non-faecal in origin and therefore represent a much less objectionable form of contamination than the typical B. coli or so-called faecal types. This view, if correct, would carry all the greater weight from the fact that aerogenes-cloacae types are of common occurrence in milk (MacConkey 1906; Orr 1908; Rogers, Clark and Davis 1914; Levine 1921; Kline 1930; Kon 1933; Zavagli 1933; Yale 1933). In an investigation by the writer (1933) of the types of coliform bacteria occurring in "raw" or unpasteurised milk at different seasons, 359 cultures of these organisms were isolated from specimens of raw milk during a winter period when the cows were confined for the greater part of the

day to byres, and 438 cultures during a summer period when the cows were at pasture and brought into the byres only at milking time. It was found that the coliform flora of the milk varied greatly according to season. Thus in winter the ratio of typical B. coli to aerogenes-cloacae types was 2·4:1, while in summer it was 0·7:1. Since typical B. coli are the predominant types in bovine faeces, it was suggested that this variation was associated with a greater possibility of faecal contamination of the milk in winter when the cows were confined to byres than in summer when they were at pasture. There remained however to be settled the question of the source of the aerogenes-cloacae types in milk and also the conditions which are responsible for their prevalence in it during summer. Accordingly, in view of the importance attached to the coliform test in the bacteriological examination of milk supplies and of the fact that coliform organisms, especially aerogenes-cloacae types, are frequently responsible for spoilage of milk and cream and defects in butter and cheese, it was decided to carry out further work for the purpose of elucidating these questions. This investigation has now been completed and forms the subject matter of Sections II. and III. of the present thesis. In the course of the work large numbers of

coliform bacteria were isolated and their characters determined. A difficulty, however, arose with regard to the classification of the organisms owing to the widely differing views held by various workers. An examination was therefore made of the systematic value of the biological characters of all the types isolated in this, and in a previous (Malcolm 1933), investigation. This study constitutes Section IV. of the thesis. It has led to the elaboration of a scheme of classification which, it is believed, enables the organisms to be classified in a satisfactory manner, and is more logical and comprehensive than any yet devised.

Prior to the commencement of the work, the chief primary sources from which coliform organisms may gain access to milk at the farm were considered, namely fodder and bedding (i.e. hay, ensilage, straw), cereal grains, feeding cakes, grass, soil and bovine faeces. As previously mentioned, it is generally accepted that the B. coli are the prevalent types in human and animal faeces but in absence of recent faecal contamination they do not occur to any extent on vegetation and cereal grains and in the soil and water supplies. The aerogenes-cloacae types, according to many workers, are seldom present in faeces but frequently occur on vegetation, ensilage,

cereal grains and in soil. As regards the latter, however, some investigators are of the opinion that such types are almost invariably present in faeces but in small numbers. These findings with regard to the distribution of aerogenes-cloacae types in nature were borne in mind in considering the primary sources from which the organisms may gain access to milk. The fact, however, that aerogenes-cloacae types are most prevalent in milk in summer (Malcolm 1933; Wilson 1935) enabled a number of these sources of contamination to be eliminated. Thus at this season the cows are at pasture and brought into the byres only at milking time. Consequently they are not provided with fodder and bedding and there is little or no possibility of the milk being contaminated with hay, ensilage and straw, or dust from these materials. It is possible that contamination of the coats of the cows may occur in summer from contact with pasture plants, but as the animals move freely about the fields, they themselves in many, if not in most, instances may be responsible for the presence of coliform bacteria on the plants. In any case Wilson (1935) found that aerogenes-cloacae types were comparatively seldom present on what he termed the "soil-contaminated materials" - straw, hay, grass, decaying leaves, water and swedes. He frequently obtained them

from cereal grains, meals and feeding cakes, but there is little or no possibility of milk being contaminated from these sources in summer as at this season the cows, being at pasture, receive only very small quantities, if any, of such foods. On the other hand, the coats of the cows may be contaminated in summer with mud. This, however, is more likely to happen in winter than in summer, as in winter the fields, roads and gateways where the cows go for exercise, are generally in a wetter and muddier condition. In any case such mud is as a rule heavily contaminated with faeces, and the coliform organisms present will in all likelihood be of faecal origin. In this connection it must be remembered that Houston (1897-1898, 1899-1900, 1900-1901, 1902-1903), Burke-Gaffney (1932), Bardsley (1934) and others, have found that coliform organisms are frequently absent from soils which are free or relatively free from faecal contamination. Moreover, Bardsley (1934) found that where coliform organisms occur in such soils in Britain, they are largely of B. coli and intermediate types (31.0 per cent. B.coli and 66.4 per cent. Intermediate), aerogenes types being comparatively rare. Wilson (1935) in discussing the comparative absence of aerogenes-cloacae types from soil-contaminated materials such as straw, hay, grass, water and swedes, states that this "to some extent confirms Bardsley's observations on

the infrequency of aerogenes-cloacae organisms in the soil of this country". Although dust is more prevalent in summer than in winter, it arises chiefly from roads, especially untarred farm roads, and not from fields carrying their summer crops. Such road dust is almost certain to contain particles of faeces. Wilson (1935) suggests that milk may be contaminated with aerogenes-cloacae types in summer from the dust from feeding substances, but as previously stated, in summer, such foods are either not given at all or only to a very limited extent.

In view of these facts it was decided to investigate whether the remaining primary source of coliform contamination of milk, bovine faeces, may not be responsible for the occurrence of aerogenes-cloacae types in milk. Although it is generally stated and accepted that such organisms are seldom present in bovine faeces, nevertheless, this view is not supported by the results of some workers. Thus Allen (1923) found that 8.4 per cent. of the total bacteria present in fresh bovine faeces were of B.aerogenes type and Dorner (1926) showed that at certain periods B. aerogenes types appeared to predominate strongly but at other periods B. coli. Moreover Ford (1927) stated that B. aerogenes occurs chiefly in bovine faeces, while according to Sherman (1935) B. aerogenes types occur constantly

in animal faeces though in relatively small numbers. It is therefore possible that the incidence of aerogenes-cloacae types in bovine faeces may be similar to what Cruickshank and Cruickshank (1931) and Hay (1932) found with human faeces, namely that such organisms are almost constantly, though scantily present. If that is the case, this material may constitute an important primary source of these types in milk, for although they may be present only in small numbers in the faeces, their relatively high powers of resistance may enable them to become enriched in the milk, in the utensils and elsewhere. Accordingly in the first place an investigation was carried out to determine whether aerogenes-cloacae types were frequently present in bovine faeces.

Specimens of fresh faeces, both from cows confined to byres in winter and from cows at pasture in summer, were examined bacteriologically to determine by the ordinary methods of isolation the various types of coliform bacteria which are present under these varying conditions. The cultures thus isolated were found to consist chiefly of B. coli types, the most prevalent organisms being B. coli communior (MacConkey's type no.71) and B. coli communis (MacConkey's type no.34), as in faeces of human origin. (More than half of the cultures

were of B. coli communior type). Comparatively few aerogenes-cloacae cultures were obtained. There was no significant variation in the types according to winter and summer conditions. Having regard to these results it was considered that the failure to obtain aerogenes-cloacae types more frequently might be due to the small number of these organisms in the faeces as compared with B. coli types. The investigation was therefore continued to determine whether the former could be obtained more frequently from bovine faeces by enrichment methods of isolation. Various methods were used to enrich the organisms prior to plating, e.g., their cultivation in peptone water containing brilliant green or in Koser's fluid citrate medium. It was found that more than one subculturing - two in the case of brilliant green peptone water and three in the case of Koser's medium - in the enrichment medium was necessary to eliminate the typical B. coli. Proteus types grew well in Koser's medium but could be checked by the addition of 1:1000 to 1:2000 copper sulphate. By employing such enrichment methods it was found that organisms of aerogenes-cloacae types could be isolated from almost all the faecal specimens and there was no significant variation in their incidence in the faeces with summer and winter conditions. B. cloacae (MacConkey's

type no.108) and B. aerogenes (MacConkey's type no.103) were the types most frequently obtained.

According to the results of these investigations the typical B. coli predominate in bovine faeces but the aerogenes-cloacae types are practically always present, though normally in small numbers. The occurrence of aerogenes-cloacae types in almost all the specimens of bovine faeces showed that this material may constitute an important primary source of such organisms in milk. Moreover, this finding was supported by the observation that in the winter time the same ratios hold for aerogenes-cloacae types to B. coli types in the faeces and in the milk. With regard to the incidence of the various types in the specimens, however, an explanation had yet to be obtained for the fact that the ratio of aerogenes-cloacae types to B. coli was much higher in summer with the milk than with the faeces, and the ratio was also much higher with summer milk than with winter milk. In considering this variation in the coliform flora of milk according to summer and winter conditions, it was thought that the relatively high incidence of aerogenes-cloacae types in summer milk may be due to the fact that while under both summer and winter conditions faecal contamination of the milk may occur, in winter there is little or no growth of

any coliform types in the milk so that the coliform flora resembles that of faeces, whereas in summer there is considerable growth and multiplication of the aerogenes-cloacae types, but not of the B. coli, and as a result the former become enriched in the milk. There were grounds for this supposition, as it had been found in a previous investigation (Malcolm 1933) that (a) the average bacterial content of specimens of milk from a group of farms is as a rule much higher in summer than in winter, (b) the incidence of coliform organisms in the specimens, as shown by the coliform test, is much higher in summer than in winter, and (c) there is a high correlation between the average bacterial content of a series of such specimens, the proportion of coliform-positive samples and the mean of the daily minimum and maximum atmospheric temperatures. At the time it was suggested that this correlation was probably due largely to the influence of temperature on the rate of multiplication of the bacterial contaminants (including coliform organisms) both in milk, which has not been properly cooled, and in utensils which have not been thoroughly sterilised.

In view of these facts it was decided to carry out a further investigation to find whether aerogenes-cloacae types might not become enriched in milk held at

low temperatures such as prevail in dairy practice. Cultures of various types of coliform bacteria - B.coli, B.cloacae, B.oxytocus and B.aerogenes - were grown in partially or completely sterilised milk for 24 hours at 17°C., the bacterial numbers in the milk before and after incubation being determined by the plating method. In all instances the aerogenes-cloacae types multiplied more rapidly than the B. coli. There was a possibility that different results might have been obtained if raw milk had been used instead of sterilised, since raw milk, unlike heat-sterilised milk, has bacteriostatic or bactericidal properties, which may have a greater effect on aerogenes-cloacae types, than on B. coli. The experiment was therefore repeated with raw instead of sterilised milk. It was found that while the raw milk had a marked bacteriostatic action on certain coliform organisms, both of B. coli and aerogenes-cloacae types, the aerogenes-cloacae were generally affected to a much less extent than the B. coli and as a rule multiplied much more rapidly. As the plate counting method, used in these experiments to determine the rate of bacterial multiplication, would give unreliable results if the organisms occurred in clusters in the milk, microscopic examination in many instances was made of the primary bacterial suspensions

with which the milk was inoculated and of the inoculated milk after 3 days incubation at 17°C. However, there was little or no tendency to clumping either with the sterilised or with the raw milk. Moreover, the initial plate counts of the milk cultures agreed fairly closely with the estimate based on the count to which the primary suspensions of the inoculums had been standardised by the opacity method.

There was a possibility that the relative rates of growth and multiplication of the different coliform types might vary widely according to whether the organisms were living in pure cultures or in mixed cultures of coliform types. Experiments were therefore carried out to determine whether aerogenes-cloacae types would multiply more rapidly than B. coli, when mixed cultures, each consisting of a B. coli and an aerogenes-cloacae type, were grown in sterilised milk held at 17°C. for 24 hours. The relative rates of multiplication of the two coliform types in each culture was determined. It was found that in most instances the aerogenes-cloacae types multiplied more rapidly than the B. coli, - often much more rapidly - i.e., the behaviour of the organisms in mixed culture was similar to that in pure culture.

The question was then considered as to whether

temperature had any marked influence on the results. Mixed cultures of different coliform types, as in the previous experiment, were grown in milk for 24 hours, not only at 17°C. but also at 22°C., 30°C., and 37°C. The results showed that at 22°C., as at 17°C., the aerogenes-cloacae type multiplied as a rule more rapidly than the B. coli, but at 30°C. and 37°C. the reverse was the case. Thus the relative rates of multiplication of aerogenes-cloacae and B. coli types in milk varied with temperature.

The investigation had thus shown (1) that aerogenes-cloacae types were almost always present, though in small numbers, in bovine faeces and (2) that they multiplied more rapidly than B. coli types in milk held at 22°C. or lower temperatures. From these results the inference was drawn that milk, which is contaminated with bovine faeces, and cooled and held at a temperature of 17°C. or 22°C., may ultimately contain a coliform flora consisting largely of aerogenes-cloacae types. To determine whether this was the case, specimens of bovine faeces were inoculated into raw milk and the cultures kept at 17°C. for 36 hours. Plates of MacConkey's agar were stroked in series from each milk culture and a large number of colonies picked off from each set of plates and typed. It was found that the aerogenes-cloacae types occurring in

the faeces became enriched in the milk, the coliform flora of the milk at the end of the incubation period frequently consisting chiefly of these types.

From the results of these investigations, the conclusion is drawn that the greater incidence of aerogenes-cloacae types in summer milk in Scotland compared with winter milk, as found in a previous investigation, may be due to the fact that when milk is contaminated directly or indirectly with faeces, these types become enriched at the temperatures of storage commonly prevailing in summer. Such enrichment does not occur in winter, the holding temperature of the milk being as a rule so low that there is little or no proliferation of any coliform types. Consequently the relative proportions of the various coliform types in winter milk tend to remain similar to those in the faeces.

In these investigations large numbers of coliform bacteria were isolated. The biological characters of the organisms were examined to enable them to be typed and placed in their proper groups - B. coli, B. cloacae, B. oxytocus, B. aerogenes, etc. There is, however, considerable difference of opinion as to how coliform bacteria should be classified and therefore the question arose as to which classification should be adopted. In

the classifications of the earlier investigators (Dyar and Keith 1894, Smith 1895, Refik 1896, Gordon 1897, Grimbert and Legros 1900, Durham 1900-1901, Ford 1901, Houston 1902-1903 and 1905, Jordan 1903, Savage 1906), comparatively few biological characters are employed and as a result only a small number of types or groups are differentiated. Such classifications are by no means complete and are too crude for use. For example, Durham simply arranged the lactose-fermenting types of coli-typhoid organisms in three groups: B. coli communis - motile and non-sucrose-fermenting; B. coli communior - motile and sucrose-fermenting; and B. lactis aerogenes - non-motile and polysaccharide-fermenting. Houston (1905) grouped the cultures he isolated from milk on the so-called "flaginac" basis, namely "fl" - production of fluorescence in neutral red broth; "ag" - fermentation of lactose with formation of acid and gas; "in" - production of indole; "ac" - milk acidified and curdled. He also noted whether the cultures fermented sucrose and liquefied gelatin.

The first comprehensive classification of coliform bacteria was that of MacConkey (1905, 1906, 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, depending on 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 sub-groups according to their sucrose and dulcitol reactions and subdivided the sub-groups into a number of types or varieties according to the other characters mentioned. Unfortunately, MacConkey's sub-group criteria - sucrose and dulcitol reactions - do not correlate very highly with certain features shown by later workers to be of great systematic value, i.e., the Voges-Proskauer, Koser and indole reactions. In fact the sucrose and dulcitol reactions are of no value for the differentiation of the typical B. coli from aerogenes-cloacae types, both B. coli and aerogenes-cloacae types occurring in each of MacConkey's sub-groups. While MacConkey employed the Voges-Proskauer and indole reactions for systematic purposes he attached no special importance to them, but simply included them in a series of biochemical characters for the differentiation of types. 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. One great 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 sub-groups correlated more

highly with the indole, Voges-Proskauer and gelatin reactions, than did the sucrose-dulcitol sub-groups. 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 Harden and Walpole (1906), 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 carbo-

hydrate 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 (1916a, 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 sub-groups 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,

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.

e.g., non-motility, encapsulation, 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 sub-groups:

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 sub-group 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 sugars fermented and non-liquefying, e.g., B. coli anaerogenes.

Subsequent to the publication of Mackie's classification, a valuable biological feature for the differentiation of coliform types was discovered as a result of the work of Brown (1921) and Koser (1923, 1924, 1926, a,b,c). These investigators showed that aerogenes-cloacae types were capable of utilising the citrate radicle as the sole source of carbon, while B. coli types 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, Koser 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. These Koser-positive, methyl-red-positive, Voges-Proskauer-negative soil types were dissimilar to aerogenes-cloacae types, but resembled B. coli, in that they were unable to grow in media containing uric acid unless some other source of nitrogen was available (Koser 1918, Chen and Rettger 1920).

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: see also Section IV.), they are highly characteristic properties of various important sub-groups 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; see also Section IV.). Also, misleading results may be obtained under such conditions owing to the liquefaction being due merely to the action of endoproteases 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 sub-division 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 th 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 and Sandholzer (1935) showed that intermediate coliform types varied with regard to Koser reaction, sulphuretted hydrogen and indole production and cellobiose and ∝-methyl-glucoside fermentation, and concluded that intermediate coliform types were so heterogeneous in character that their inclusion in a separate genus was hardly justified.

Wilson (1935) subdivides coliform organisms according to their methyl-red, Voges-Proskauer, Koser citrate, Eijkman, indole and gelatin reactions. The Eijkman reaction depends upon the ability of the organisms to produce gas in dextrose broth when incubated at 46°C.,

B. coli types giving positive reactions and aerogenes-cloacae negative. Investigators are by no means unanimous as to the reliability of this test, although Wilson (1935) claims that the discrepancies in the results of the various workers have been due to failure to maintain a constant temperature of 43 to 45°C. in the medium during incubation.

It is evident from a comparison of Bergey's classification with that of Mackie, Wilson 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. 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. Routine examination of water and milk for coliform bacteria by Public Health Authorities is directed solely to detecting the presence of these organisms, and in the case of water, ascertaining whether they are "typical" B. coli 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 for the systematic study of the biological characters of the group, a comprehensive classification of types is essential. It is also necessary in investigations (such as the present one) of the types of coliform bacteria occurring in faeces, milk, etc. Consequently in carrying out the examination of the coliform flora of bovine faeces, it was decided to make at the same time a detailed study of the biological characters of all the coliform cultures isolated from the bovine faeces and also of those isolated in a previous investigation (Malcolm 1933) from specimens of milk. It was thought that in this way information might be gained with regard to the value of these features as differential criteria, and a satisfactory method of classification be obtained.

One of the chief difficulties in the classification of coliform bacteria arises from the fact that while having a number of characters in common, they show wide variation with regard to certain others, the true value of which for differential purposes is not known. In order to overcome this difficulty it was decided to assess the systematic value of each character, not only on its

morphological or biological significance, but also on the degree to which it was correlated with other characters. The latter principle of classification has not hitherto been carried out to any considerable extent, except by Levine (1918). Accordingly the characters of certain well recognized types of coliform bacteria, namely B. coli, B. aerogenes, B. cloacae and B. oxytocus, were examined and the most outstanding features determined. A study was then made of the biological characters of all the cultures, 1,636 in number, which had been isolated from milk and bovine faeces, and the degree of correlation between the various characters ascertained.

It was found that there are numerous types of coliform bacteria. These types are so closely interlinked in characters and in relations to environment as to render it undesirable to make an arbitrary division of the group into two or more genera. However, for systematic purposes and to enable types to be identified more readily, the group may be subdivided into a series of sub-groups by means of four biological characters, namely the Voges-Proskauer, Koser citrate, inositol and indole reactions. These differential criteria are not only reliable and outstanding biological features but they correlate almost perfectly with various other characters, and by their use the

group may be divided into particularly well defined sub-groups. Thus the Voges-Proskauer reaction not only affords an index of the gas ratio (CO_2:H_2) of a coliform type, but also of its acid-producing power or methyl-red reaction. As regards the cultures examined, there was an almost perfect correlation between the Voges-Proskauer and methyl-red reactions. Moreover, the Voges-Proskauer-positive cultures were with rare exceptions Koser-positive. The Koser reaction was found to give reliable information with regard to the viability of the organisms, Koser-positive types being more resistant to the antiseptic action of brilliant green than Koser-negative. The greater vigour of the Koser-positive types was further indicated by the fact that not only could they attack the citrate radicle, but they frequently fermented inositol and in many instances produced thick mucoid colonies and became encapsulated. Moreover, Koser-negative types were in nearly all instances typical B. coli, i.e., they gave Voges-Proskauer negative, inositol-negative and indole-positive reactions. There was a very high negative correlation between the inositol reaction and motility; and an almost perfect positive correlation between the inositol and adonitol reactions with the exception of the adonitol-positive typical B. coli, these latter, like all other Voges-

Proskauer-negative, Koser-negative coliform types, were in all instances inositol-negative. Inositol-fermenters were also found to be Koser-positive, sucrose-positive and raffinose-positive; they were frequently encapsulated and formed thick mucoid colonies. The indole reaction correlated very highly in a negative sense with the Koser reaction; but indole-positive, inositol-positive types were Koser-positive. Thus indole-negative types were Koser-positive, while indole-positive types were as a rule Koser-positive or negative according to whether the inositol reactions were positive or negative.

Selecting these four characters - the Voges-Proskauer, Koser, inositol and indole reactions - as differential criteria, a system of classification was formulated by means of which the cultures which had been isolated, were arranged in eight sub-groups. The prototypes of five of these sub-groups were of classic coliform types, namely B. coli communis, B. friedländeri, B. cloacae, B. oxytocus and B. aerogenes. No named types belonged to the other three sub-groups, though organisms of these sub-groups have also been obtained by MacConkey (1905, 1909), Mackie (1921), Koser (1924, 1926), Bardsley (1926, 1934) and others.

S E C T I O N II.

THE TYPES OF COLIFORM BACTERIA IN BOVINE FAECES.

Specimens of bovine faeces, both from cows confined to byres and from cows at pasture, were examined bacteriologically, with the following objects: (1) to determine by the ordinary methods of isolation the various types of coliform bacteria which are present in bovine faeces under these varying conditions, (2) in the event of aerogenes-cloacae types being rarely obtained by these methods, to attempt to isolate them more frequently by enrichment methods, and (3) to study the biological reactions of the cultures of coliform organisms isolated.

I. The Types of Coliform Bacteria isolated from bovine faeces by Ordinary Methods.

One hundred and fourteen specimens of bovine faeces were examined. The specimens were taken at different periods of the year, eighty-one being obtained during a winter period extending from November to April, when the animals were confined to byres for most of the day, and thirty-three during a summer period extending from May to October, when the cows were at pasture for the whole or greater part of

the day. Most of the specimens were obtained from six cows, and were procured from the centre portions of fresh droppings.

The specimens were examined within a few hours of evacuation, the examination being carried out as early as practicable in view of the possibility of certain organisms dying off rapidly in such materials (Browning 1918). If the faeces could not be examined within 6 or 8 hours, they were treated at once with Teague and Clurman's glycerine and salt solution (see Browning 1918).

Methods of isolation.

An emulsion of the faeces was prepared in sterile water. At first one large loopful of the specimen was mixed with approximately 5 ml. sterile tap water, the diameter of the loop being about 4 mm. Later, when it was observed that no coliform cultures were obtained from some specimens, a much denser emulsion was prepared, six to ten loopfuls of faeces being added to the sterile water.

The following methods of isolation were employed: (a) stroked plates of bile-salt lactose agar, and (b) preliminary enrichment in bile-salt lactose bouillon.

Stroked plates of neutral-red bile-salt lactose agar (Muir and Ritchie 1937). One large loopful of the faecal emulsion was stroked on a bile-salt agar plate and in-

cubated at 37°C. for 24 hours. Typical colonies (at first six but later in the investigation three) were transferred to Durham's fermentation tubes of bile-salt lactose bouillon. Subcultures which failed to produce acid and gas within 14 days at 37°C. were discarded. The others, as soon as both acid and gas were apparent in the fluid cultures, were subcultured on bile-salt agar plates. Single colonies were again subcultured into bile-salt lactose bouillon, and if producing acid and gas, transferred to agar slopes. Replating from a broth culture was considered to be advisable owing to the difficulty often experienced in separating cultures of coliform organisms from mixed cultures. This procedure has been recommended for similar reasons by Ruchhoft, Kallas, Chinn and Coulter (1931). Coliform cultures were not obtained by this method from sixteen specimens, but they were isolated from twelve of these specimens by other methods which were being used at the time. Thus only four specimens failed to yield cultures by any of the isolation methods employed. It was possible that in some instances coliform colonies had been overlooked on the bile-salt agar plates, and so the practice was finally adopted of keeping the plate cultures from which colonies had been taken, for 6 or 7 days. If the bouillon subcultures failed to produce acid and gas within that time, fresh subcultures were made from other

colonies. Three of the four specimens which yielded no coliform cultures were examined in the early stages of the work before this practice was followed. The presence of "spreading" colonies, chiefly Proteus types, on the plates occasionally made the isolation of coliform cultures difficult. It is noteworthy that Dorner (1926) frequently failed to obtain any coliform strains from specimens of bovine faeces, especially with certain cows. As a rule cultures from three colonies of coliform organisms were isolated by this method from each specimen. If the colonies differed in appearance, subcultures were made from each type. The total number of cultures obtained was 262.

Growth in bile-salt lactose bouillon before stroking on bile-salt lactose agar plates. Several loopfuls of the faecal emulsion were added to a Durham's fermentation tube of bile-salt lactose bouillon and incubated at 37°C. When acid and gas were produced (usually within 18-24 hours), the culture was stroked on a plate of bile-salt agar. The subsequent procedure was the same as in the first method, but only one colony was inoculated into lactose bouillon, and as a result not more than one culture was isolated from each specimen. (Where the specimen contained several types of coliform bacteria, the

type isolated would depend to some extent on the relative numbers of the various types in the faeces and also on their relative rates of growth in the bile-salt bouillon). This method was not used at the outset of the work, but was employed with the plating method (see above) in the examination of the last eighty-five specimens, i.e., the thirty-three summer specimens and fifty-two of the eighty-one winter specimens. Coliform cultures were obtained by its use from eighty of these specimens, including seven from which none was obtained by the previous method. The total number of cultures isolated was eighty.

Method of identification of cultures isolated.

Stained smears were prepared from the agar-slope cultures and examined microscopically to determine whether the organisms were of coliform type, i.e., non-sporing, Gram-negative and rod-shaped. Cultures which had not these characters and failed to grow under aerobic conditions were discarded. Living preparations from peptone-water cultures were examined for motility. The following biochemical reactions were also determined: fermentation of lactose, glucose, sucrose, dulcitol, adonitol, inulin, raffinose and inositol; action on milk; the methyl-red and Voges-Proskauer reactions; production of indole; liquefaction of gelatin and ability to grow and produce

turbidity in Koser's fluid citrate medium (Koser 1924). The various tests were carried out in the manner described on pages 94 to 98.

Results.

Three hundred and forty-two cultures of coliform bacteria were isolated by these methods. The cultures obtained by both the methods corresponded very closely in type, and consequently the results obtained by both are combined in Table 1, page 146, which shows the number of cultures isolated during the winter and summer periods, and during the whole period. The cultures are arranged in sub-groups according to the following reactions:- (a) Voges-Proskauer reaction, (b) growth in Koser's fluid citrate medium, (c) fermentation of inositol and (d) production of indole. This method of grouping is supported by the facts given in Section IV, page 93.

It will be seen from Table 1, that in each of the periods most of the cultures belonged to sub-group 1, the typical B. coli. Of the 342 cultures isolated, 330 belonged to this sub-group and only twelve to the other sub-groups. With regard to these B.coli cultures (sub-group 1) the following types of MacConkey (MacConkey 1905, 1909) were obtained, the figure in brackets indicating the number of cultures of each isolated: nos. 71 or B. coli

communior (185, i.e., more than half of the total number of cultures isolated), 34 or B. coli communis Escherich (69), 1 (23), 72 (14), 35 (11), 4 (8), 5 (5), 107 (5), 106 (3), 2 or B. acidi lactici (3), 100 (2), 33 (1), and an atypical strain (1). In faeces of human origin a similar condition exists, the most prevalent coliform organisms are typical B. coli, and of these nos.71 and 34 are most frequently found (Muir and Ritchie 1937; Cruickshank and Cruickshank 1931; Hay 1932). The twelve other cultures belonged to the following MacConkey types, the figure in brackets indicating the number of each type obtained: nos.103 (3), 65 (3), 7 (1), 67 (1), 68 (1), 73 (1), 74 (1) and 108 (1). Four of these cultures were obtained from the one specimen.

Cultures of aerogenes-cloacae and other Koser-positive types were therefore seldom obtained from bovine faeces by the ordinary methods of isolation. The question then arose as to whether this was due to their rare occurrence in bovine faeces or to the fact that they were present in such small numbers as to render their isolation difficult by the ordinary methods. Consequently the work was continued with the object of determining to what extent they could be obtained from bovine faeces by using methods of isolation suitable for their enrichment.

II. The Incidence of Aerogenes-cloacae and other Koser-positive types in bovine faeces, as determined by the use of enrichment methods of isolation.

One hundred specimens of bovine faeces were examined, Koser-positive cultures being isolated from ninety-five of them by various enrichment methods. Fifty of the specimens were obtained during summer when the cows were at pasture, and fifty during winter when the animals were confined to byres. The specimens were from various cows on four farms. The same procedure was adopted with regard to collection of the specimens as in the previous work.

The following methods of isolation were employed: (a) preliminary enrichment in Koser's fluid medium, (b) preliminary enrichment in a fluid medium containing brilliant green, (c) preliminary enrichment in Koser's fluid medium plus copper sulphate, and (d) stroked plates of Simmons' citrate agar.

Enrichment in Koser's fluid citrate medium prior to stroking on a bile-salt lactose agar plate. A tube of Koser's fluid medium was inoculated with the faecal emulsion. (For this primary culture at first 20 ml. of Koser's medium and ten loopfuls of the faecal

emulsion were used, but later only 10 ml. of the medium and three large loopfuls of the faeces). When growth was apparent, usually after 18 or 24 hours' incubation, a subculture was made into another tube of Koser's medium, a smaller amount of inoculum being used than that employed for the primary culture. If the second tube showed turbidity (a positive Koser reaction), another subculture was made similarly in the same medium. When the third Koser tube gave a positive reaction, the culture was tested in bile-salt lactose bouillon, and if acid and gas were produced it was stroked on a bile-salt lactose agar plate. A colony from the plate was subcultured in bile-salt lactose bouillon and in Koser's citrate medium. If the reactions in both cases were positive, the culture was again stroked on bile-salt agar; a colony was tested in lactose bouillon and in Koser's medium and then a subculture made on an agar slope. If a culture failed after either the first or second plating to give positive reactions in both lactose bouillon and Koser's medium, fresh subcultures were made from other colonies on the plate. At first an incubation temperature of 37°C. was used in this and the other enrichment methods, but later it was reduced to 30°C., as it was found that colonies of Koser-positive types developed to a greater extent at

this temperature than colonies of B. coli types.

As a rule no attempt was made to isolate more than one culture from each specimen by this method. In thirty-eight specimens no cultures were isolated, in spite of the fact that in most instances cultures were obtained by one or more of the other enrichment methods. Failure to obtain cultures was due to the following factors. (1) Cultures were frequently obtained which proved on replating and further examination to be mixed cultures of Koser-negative coliform types and Koser-positive non-coliform (especially Proteus) types. This difficulty was overcome to some extent by retaining the plate cultures for some time after subcultures from the colonies had been made. The subcultures were tested as usual in lactose bouillon and Koser's medium, replated and tested again in lactose bouillon and Koser's medium. Then, if they failed to give positive reactions in both these media, fresh subcultures were made from other colonies on the primary plates. However, as the work progressed and experience was gained, Koser-positive colonies could be picked off from the plates with a greater degree of certainty, especially if the plates were kept for 24 hours at 30° C. and then for several days at room temperature before the colonies were examined.

(2) Trouble was experienced in isolating cultures from some of the plates owing to the abundant growth of Proteus types, which had not been eliminated by the subculturing in Koser's medium. It was found that growth of such organisms in Koser's medium could be prevented by the addition of copper sulphate (Allison and Ayling 1929) (see enrichment method (c).) All the Koser-negative cultures of coliform bacteria obtained during this section of the work, i.e. by these enrichment methods, were discarded and are therefore not included in Tables 2, (page 147) and 3 (page 148).

Enrichment in brilliant green peptone-water before plating in bile-salt lactose agar (Muir and Ritchie 1937, Browning 1918). Preliminary experiment had shown that the addition of 0·7, 1 and even 1·5 ml. of a 1 : 10,000 solution of brilliant green to 10 ml. amounts of peptone-water, which had been inoculated with bovine faeces, was insufficient to eliminate B. coli types, a fact which was probably due to the effect of the large quantity of extraneous matter introduced with the inoculum. Consequently the practice was adopted of subculturing twice in the brilliant green medium. As primary cultures, several large loopfuls of the faecal emulsion were inoculated into two tubes containing 10 ml. peptone-water plus 0·7 and 1·0 ml. of a 1 : 10,000 solution of brilliant

green respectively. Then, after 18-24 hours' incubation at 30°C., a subculture was made from each into peptone-water containing the same concentration of the dye as the primary culture. At the same time plates of bile-salt lactose agar were stroked. The secondary cultures in peptone water were incubated for 24 hours and then stroked on bile-salt agar. There were therefore two pairs of plates, one from the primary and the other from the secondary cultures. The plates were incubated at 30°C. and one colony from each was tested in bile-salt lactose bouillon and Koser's fluid citrate medium. If positive reactions were obtained the culture was replated on bile-salt lactose agar, a colony was subcultured from it and tested in bile-salt lactose bouillon and Koser's medium. If the reactions were positive, the culture was transferred to an agar slope. Only one culture as a rule was isolated by this method from each primary and secondary culture. Koser-negative strains were occasionally obtained, but were discarded. As with the Koser fluid enrichment method, the plate cultures were retained for some time, so that in the event of the subcultures from the colonies proving to be Koser-negative other subcultures could be made.

In the examination of the last fifty specimens (the winter specimens) the method was modified as follows.

Two primary cultures were made in peptone-water containing 1.0 ml. of the dye, the inoculum being in the one instance three large loopfuls of the semi-fluid faecal specimen and in the other twice or three times that quantity. After 18-24 hours' incubation at 30°C. secondary cultures were made from each in peptone-water containing 1.0 ml. of the dye. Plates of bile-salt agar were stroked only from the secondary cultures. The subsequent procedure was the same as previously detailed. Cultures were not obtained by the various brilliant green enrichment methods from nineteen of the 100 specimens, although they were obtained from fourteen of these nineteen specimens by one or more of the other enrichment methods.

Enrichment in Koser's fluid medium containing copper sulphate prior to plating on bile-salt agar. Trouble had been experienced in isolating Koser-positive cultures by enrichment in Koser's medium owing to the abundant growth of Proteus types. To overcome this difficulty a sterile copper sulphate solution was added to the medium after sterilisation (Allison and Ayling 1929). At the outset 1 ml. of a 1 : 100 solution of copper sulphate was added to 10 ml. of Koser's medium, giving a concentration of copper sulphate in the medium of 1 : 1000, but in the later work only 0.5 ml. was added,

yielding a concentration of 1 : 2000. The latter procedure gave the more successful results. Cultures were not obtained by this method from fifty-seven specimens.

Stroked plates of Simmons' citrate agar.

Attempts were made to isolate Koser-positive cultures by stroking plates of Simmons' citrate agar with loopfuls of the faecal emulsions (Simmons 1926). This method, however, was discarded, as it was found that Proteus types grew well on the plates and also that colonies of Koser-negative coliform organisms were frequently present. The growth of the latter on the citrate agar was probably promoted by the presence of extraneous organic substances introduced along with the inoculum.

Results.

Of the 100 specimens examined by these enrichment methods, Koser-positive coliform cultures were obtained from ninety-five. The failure to obtain them from five specimens was possibly not due to their absence from the faeces but to the fact that the proper colonies had not been subcultured from the plates. As already mentioned, in many instances subcultures from the colonies gave positive Koser and lactose reactions, but on replating they proved to be mixed cultures of Koser-negative coliform

types and Koser-positive non-coliform types, most frequently Proteus.

In Table 2, page 147, is shown the number of Koser-positive coliform cultures isolated during the various periods, the cultures being arranged according to the same biochemical reactions as in Table 3, page 148. As no correlation could be observed between the frequencies of the types obtained and the various enrichment methods of isolation, the cultures are not grouped according to method of isolation used. It will be seen from the table that in each of the periods most of the cultures belonged to sub-groups 6 and 8, i.e., they were of B. cloacae and B. aerogenes types. The latter predominated in the summer and the former in the winter. However, there appeared to be no significant difference between their relative frequencies for the two periods, and almost the same number of cultures of each (eighty-three in the case of B. cloacae and eighty-six in the case of B. aerogenes) were obtained during the whole period. It is noteworthy that while eighty inositol fermenters and forty-eight inositol non-fermenters were isolated during the summer, forty-four of the former and eighty-one of the latter were obtained during the winter, i.e., practically the numbers were reversed. No reason

can be given for this variation.

The following are the types of MacConkey which were obtained, the figure in brackets being the numbers of cultures of each isolated: no.108 (56, two being of anaerogenes type), 103 (37), 67 (18), 73 (15), 98 (13), 109 (13), 7 (10), 65 (10), 102 (8), 69 (6), 75 (6), 101 (6), 66 (5), 104 (4), 36 (3), 68 (2), 97 (2), 8 (1), and 74 (1). There were thirty-seven atypical strains. Eight of these were B. coli types (MacConkey's types, nos. 71, 1 and 5) giving atypical (i.e. positive) Koser reactions.

Biological characters of the cultures. The biochemical reactions and motility of the cultures isolated are given in Table 3, page 148, all the cultures shown in Tables 1, and 2, being included. The lactose, glucose mannitol, raffinose and methyl-red reactions have been omitted from the table for the following reasons. The lactose, glucose and mannitol reactions were positive in all instances. The raffinose reactions corresponded very closely with the sucrose. The methyl-red reactions as a rule were positive when the Voges-Proskauer were negative, and they were negative when the Voges-Proskauer were positive. The majority of the cultures which were exceptions to this rule were indole-negative, Voges-Proskauer-positive types, giving as a rule neutral methyl-red re-

actions, as was noted in the previous investigation (Malcolm 1933). The gelatin reaction has been omitted, as it is not a satisfactory criterion for the differentiation of coliform types. See page 135. It is, however, noteworthy that a fairly high proportion of cultures of classic gelatin liquefying types, e.g., MacConkey's types, nos. 65, 73, 97, 102 and 108, failed to liquefy gelatin even after prolonged incubation at 37°C. or lower temperatures. Thus, with regard to the forty-seven cultures of these five types isolated by the enrichment methods during the summer period, fifteen failed to liquefy gelatin.

The biological characters of coliform organisms are fully discussed in Section IV, page 93, in dealing with cultures isolated not only from bovine faeces but also from other sources. A brief statement however is given here with regard to the characters of the faecal cultures alone, i.e., the cultures included in Table 3. The Voges-Proskauer-negative cultures were in most instances Koser-negative (330 out of 393), inositol-negative (372 out of 393) and indole-positive (352 out of 393). The Voges-Proskauer-positive cultures were always Koser-positive, and most frequently indole-negative (175 out of 202); 111 were inositol-positive and ninety-one inositol-negative. The Koser-negative cultures were invariably

Voges-Proskauer-negative, inositol-negative and indole-positive, i.e., they were in all cases typical B. coli. The majority of the Koser-positive cultures were Voges-Proskauer-positive (202 out of 265) and indole-negative (216 out of 265); 133 were inositol-negative and 132 inositol-positive. As regards the non-inositol-fermenters, the indole-negative cultures were always Koser-positive but the indole-positive cultures were most frequently Koser-negative (330 out of 347). The non-inositol-fermenting cultures were also in most instances Voges-Proskauer-negative (372 out of 463) and indole-positive (347 out of 463). Moreover, they were as a rule motile and adonitol-negative; the only cultures which were adonitol-positive and inositol-negative, were MacConkey's types, nos. 1, 2, 33 and 100. The inositol-fermenting cultures were invariably Koser-positive. This applied even to indole-positive inositol-fermenting types, e.g., MacConkey's nos.65, 97 and 101. The inositol-fermenting cultures were also in most cases Voges-Proskauer-positive (111 out of 132) and indole-negative (100 out of 132); they produced mucoid colonies and were as a rule nonmotile, adonitol-positive and sucrose-positive. The motile inositol-fermenters were chiefly atypical (inositol-positive) cultures of MacConkey's type no.108, and cultures

of type no.102. All the indole-negative cultures isolated from the faeces were Koser-positive, and most of them were Voges-Proskauer-positive (175 out of 216); 116 were inositol-negative. The indole-positive cultures were most frequently Koser-negative (330 out of 379), Voges-Proskauer-negative (352 out of 379) and inositol-negative (347 out of 379).

With regard to Table 3, the cultures of sub-group 1, i.e., B. coli types, included MacConkey's types, nos. 1, 2, 4, 5, 33, 34, 35, 71, 72, 100, 106 and 107. Most of the cultures in this sub-group were motile (292 out of 330) and only thirty out of 330 gave positive adonitol reactions. The majority of the cultures were positive in sucrose and dulcitol, and all were inulin-negative. The cultures of sub-group 6, i.e., B. cloacae types, included MacConkey's types, nos. 69, 73 and 108. Cultures of types nos. 3 and 105, which also belong to this sub-group, were not obtained. Most of the cultures (eighty-two out of ninety-one) were motile and only three fermented adonitol. They all gave positive sucrose reactions. Six of these B. cloacae cultures were atypical in that they produced indole, (See page 115). The cultures of sub-groups, nos. 2, 3, 4 and 5, were intermediate in type between the B. coli and B. cloacae sub-groups. The

cultures of sub-group 3 included MacConkey's types, nos. 7, 36, 74 and 109; those of sub-group 4 included type 101; those of sub-group 5, i.e., B. friedländeri types, included type no.68 and a type similar to no.104 but differing from it in inositol reaction. No cultures of type no.70 (sub-group 3) and no.99 (sub-group 5) were obtained. The cultures of sub-group 8, B. aerogenes types, were MacConkey's types, nos.67, 98 and 103. No cultures of type no.102 (sub-group 8) were isolated. In most instances the cultures of sub-group 8 were nonmotile. Only a few cultures (five out of ninety) failed to ferment adonitol. They all fermented sucrose. The cultures of sub-group 7, i.e., B. oxytocus types, included MacConkey's type, no.65 and a type similar to no.97, but differing from it in inositol reaction. These cultures resembled B. aerogenes, apart from the fact that they produced indole and in many cases liquefied gelatin.

DISCUSSION

Regarding the incidence of coliform types in bovine faeces, it has been definitely established by the work of numerous investigators that the typical B. coli are the most prevalent and characteristic types of coliform bacteria in faeces of human and animal origin.

Cultures of these types can be isolated from most, if not all, specimens of faeces. The results of the present investigation are in agreement with these findings. Typical B. coli cultures were obtained from 107 out of 114 specimens of bovine faeces. Failure to obtain them in seven specimens was very possibly due, not to their absence from the faeces, but to various factors which prevented their isolation, e.g., overgrowth of Proteus and other organisms in the cultures.(Dorner (1926) frequently failed to obtain coliform organisms of any type from specimens of bovine faeces). The particular MacConkey types which were as a rule obtained were those which are also found most frequently in human faeces, namely, nos.71 and 34 (Muir and Ritchie 1937; Cruickshank and Cruickshank 1931; Hay 1932).

With regard to the aerogenes-cloacae types of coliform bacteria, there is still a difference of opinion as to their incidence in human and animal faeces. According to the findings of most workers, such types are seldom present in faeces, but are the most prevalent coliform types in the soil, on plants and grains and in water. These investigators conclude that such bacteria are not normal inhabitants of the human and animal intestine, and are consequently to be regarded as non-faecal

organisms. On the other hand, other workers have frequently obtained aerogenes-cloacae cultures from faeces. (MacConkey 1905, 1909; Rogers, Clark and Lubs 1918; Allen 1923; Dorner 1926; Ford 1927; Sherman 1935; Cruickshank and Cruickshank 1931; Hay 1932). The results obtained in the present investigation are in accordance with the findings of the latter group of workers. While B. aerogenes, B. cloacae and other Koser-positive cultures were seldom obtained from specimens of bovine faeces by the ordinary methods of isolation, they were found in most specimens when special enrichment methods were used.

Accordingly it would appear that such organisms are normally present in the intestines, but in small numbers compared with B. coli types. Further, the fact that they cannot be readily isolated by the ordinary methods, in both summer and winter faeces, shows that as a rule they occur relatively scantily in faeces, no matter whether the cows are at pasture or receiving house-feeding. It was found that they could be obtained much more readily from the specimens from particular cows. Their apparently greater incidence in these specimens could not be accounted for by differences in the feeding and treatment of the cows, as such specimens were met with both in the summer and winter periods. Further,

only one or two specimens might exhibit this peculiarity out of a set of ten specimens obtained simultaneously from the same farm from cows receiving the same feeding and treatment. Dorner (1926) found that the number of coliform organisms (coli-aerogenes-cloacae types) in faeces varied greatly with individual cows, and that this variation could not be attributed to the feeding, consistency of the faeces or intestinal conditions. On the other hand, Hay (1932) found that the relative numbers of B. aerogenes types in human faeces were greater in diarrhoeal conditions.

It may be concluded that although aerogenes-cloacae types may be the most prevalent coliform organisms in soil and on fodder and grains, they are also normal inhabitants of the intestines, are commonly present in faeces, and therefore they cannot be regarded as non-faecal bacteria.

SUMMARY

1. In an examination of the coliform flora of 114 specimens of bovine faeces, 342 cultures of coliform bacteria were isolated by the ordinary methods. Of the cultures obtained, 330 (or 96.4 per cent.) were typical B. coli and only twelve (or 3.5 per cent.) were of B.

aerogenes, B. cloacae and other Koser-positive types. The most prevalent types of B. coli were MacConkey's types, no.71, or B. coli communior (185 cultures or 56 per cent.), no.34, or B. coli communis Escherich (sixty-nine cultures or 20.9 per cent.), and no.1 (twenty-three cultures or 6.9 per cent.).

2. One hundred further specimens of bovine faeces were examined by various enrichment methods, 253 cultures of B. aerogenes, B. cloacae and other Koser-positive types of coliform bacteria being isolated from ninety-five of them. Eighty-three (or 32.7 per cent.) of the cultures were of B. cloacae types and eighty-six (or 33.9 per cent.) of B. aerogenes types. It would appear, therefore, that Koser-positive coliform organisms are practically always present in bovine faeces, although they occur normally in small numbers. Various workers have found that a similar state of affairs obtains for human faeces.

3. There was no significant variation in the types of coliform bacteria in the faeces with winter and summer conditions.

4. Aerogenes-cloacae types can be enriched prior to plating either by means of (a) peptone-water containing certain concentrations of brilliant green, or (b) Koser's

fluid citrate medium. More than one subculturing (two in the case of brilliant green peptone-water and three in the case of Koser's medium) in the enrichment medium gives the most satisfactory results. Proteus types grow abundantly in Koser's medium, but are checked by the addition of 1 : 1000 to 1 : 2000 copper sulphate.

5. A very high correlation was shown between various biological characters of the faecal cultures, e.g., the Voges-Proskauer and methyl-red reactions; the Koser and indole reactions; the inositol and adonitol reactions; the inositol reaction and motility; the sucrose and raffinose reactions.

S E C T I O N III.

THE ENRICHMENT OF AEROGENES-CLOACAE TYPES in MILK held at LOW TEMPERATURES: WITH OBSERVATIONS on THE RELATIVE RATES of GROWTH of AEROGENES-CLOACAE and B. COLI TYPES in MILK at DIFFERENT TEMPERATURES.

The occurrence of aerogenes-cloacae types in practically all the faecal specimens showed that bovine faeces might constitute an important primary source of such coliform types in milk, for though they might be only scantily present in faeces, it was possible that these organisms, being relatively resistant, might become enriched in the utensils and elsewhere and in the milk. This part of the investigation was therefore carried out to find whether aerogenes-cloacae types might not become enriched in milk held at low temperatures, such as are used in dairy practice.

1. Rate of multiplication of pure cultures of coliform organisms in milk held at 17° C.

Seventy-five cultures of various types of coliform bacteria (B. coli, B. aerogenes, B. cloacae and B. oxytocus) were used in this experiment; thirty-one of these cultures had been obtained from milk, twenty-nine from

bovine faeces, six from bovine urine and nine from human sources. Twenty-two of the cultures had been in stock for several years, the remaining fifty-three were recently isolated. In all instances, before being used in the experiment, the cultures were subcultured daily at 37°C. for seven or eight days on yeastrel milk agar (Wilson 1935) slopes. Then from a 24 hours' old agar slope culture, a saline suspension was prepared; a 1/100,000 dilution of the suspension was made in sterile water; and 1 ml. of this dilution was added to 50 ml. of milk, which had been partially or completely sterilised. (Partial sterilisation was carried out by a single heating of the milk for over half an hour in a Koch's steam steriliser, followed by immediate cooling to 5°C. Complete sterilisation was carried out by the intermittent method. There was no significant variation in the results of the experiments according to the method of sterilisation employed). In the latter part of the investigation the amount of inoculum was reduced and steps were taken to ensure that in different experiments the milk would be inoculated with approximately the same number of bacteria. Thus the saline suspensions, prepared from the agar slope cultures, were standardised to contain approximately 300,000,000 organisms per ml. by the opacity method of Brown and

Kirwan (1915). Consequently, in these experiments the 1/100,000 dilution contained roughly 3,000 bacteria per ml. and the inoculated milk sixty per ml. However, in all instances the bacterial content of the inoculated milk was determined by the plating method, plates being made in yeastrel milk agar with 1 ml., 0.1 ml. and 0.01 ml. of the milk. At first the 1 ml. and 0.1 ml. plates were prepared in duplicate, and the 0.01 ml. plates in quintuplicate, (Malcolm 1932), but later, when the amount of inoculum was reduced and standardised, the 1 ml. plates were made in quintuplicate and the 0.1 ml. plates in duplicate, and no 0.01 ml. plates were used. (The 1 ml. and 0.1 ml. plates were prepared by measuring 1 ml. and 0.1 ml. of the milk into the respective Petri plates; the 0.01 ml. plates, by adding 1 ml. of the milk to 99 ml. sterile water and transferring 1 ml. of this dilution to each plate). The plates were incubated at 37°C. for 48 hours and the colonies counted.

The inoculated milk was incubated at 17°C., this being the lowest temperature that could be obtained by the low temperature incubator in use at the commencement of the experiment. (This temperature corresponds closely with the temperature at which milk of the "Tuberculin Tested" grade and ordinary milk is held and marketed in

Scotland in summer, but it is higher than the temperature frequently found in the case of "Certified" milk. In accordance with The Milk (Special Designations) Order (Scotland), 1936, "Tuberculin Tested" milk must be cooled to 60°F. or 15.5°C., and "Certified" milk to 50°F. or 10°C). After the milk had been incubated for 24 hours, another bacterial count was made. In the earlier part of the investigation dilutions of 1/1000, 1/10,000 and 1/100,000 were prepared to enable duplicate plates for 0.001 ml., quintuplicate plates for 0.0001 ml. and duplicate plates for 0.00001 ml. quantities of the milk to be made, but later when the amount of the inoculum was reduced, dilutions of only 1/100 and 1/1,000 were made, quintuplicate plates being prepared for 0.01 ml. of the milk, and duplicate plates for 0.001 ml.

The results of this experiment are given in Table 4, page 149. The initial and final counts are shown and also the ratios of the latter to the former. It will be seen from these ratios that in all instances the B. aerogenes, B. cloacae and B. oxytocus cultures have multiplied more rapidly than the B. coli, and in many cases, at much higher rates. But considerable variation in the rate of multiplication is shown by different strains of the same coliform sub-group. Thus the ratio of the

final to the initial count varies with the B. coli cultures from nineteen to 427; with the B. cloacae, from 648 to 6264; with the B. oxytocus from 829 to 8600, and with the B. aerogenes from 577 to 3905. No significant difference was observed in the rate of multiplication according to whether the culture had been in stock for several years or had been recently isolated.

There was a possibility that the numbers of coliform bacteria in the milk, as estimated by the plating method, might be affected by the occurrence of the organisms in clusters, instead of separately, in the liquid. This however did not appear to be the case with the initial counts, because, as will be seen from Table 4, when standardised suspensions were used for inoculating the milk, there was a fairly close agreement between the initial counts of the various bottles of inoculated milk, and these counts also agreed closely with the estimate based on the count to which the primary suspensions were standardised by the opacity method. Nevertheless, there was a possibility that clumping of the bacteria might occur in the milk during the period of incubation, i.e., while the organisms were growing and multiplying. To determine whether this was the case a further experiment was carried out, employing thirty-two

of the cultures used in the previous experiment, fifteen of these cultures being of B. coli types and seventeen of aerogenes-cloacae.

By the procedure already described, standardised suspensions containing approximately 300,000,000 organisms per ml. were prepared from 24 hours' old agar slope cultures. Stained smear preparations were made from these suspensions and examined microscopically to confirm the fact that the organisms did not occur in clusters. The suspensions were then diluted 100,000 times and 1 ml. amounts transferred to bottles containing 50 ml. of partially sterilised milk, as in the previous experiment. As the inoculated milk contained roughly sixty bacteria per ml., it was not possible to determine by microscopic examination whether clumping of the organisms had occurred on the addition of the inoculum to the milk, but, as previously mentioned, the fact that the plate counts agreed fairly closely with this estimate showed that clumping had not taken place.

The bottles of inoculated milk were incubated at 17°C. The period of incubation was extended to three days to allow the bacteria to multiply to such an extent that on microscopic examination of stained smears from the milk a considerable number of organisms would occur

in each microscopic field. There was the additional advantage that should there be any tendency of the organisms to form clusters, the effect of this would be much greater after 72 hours' incubation than after only 24 hours. Stained smear preparations of the milk were made after 48 hours' and 72 hours' incubation and these were examined microscopically to determine whether the organisms occurred in clusters or not.

Microscopic examination of the primary suspensions and of the milk cultures after 48 hours' and 72 hours' incubation showed that in all instances there was no tendency to clumping. The organisms occurred in the smears either singly or in pairs. Clusters were very rarely observed and these in all cases were small in size. Accordingly, little or no importance can be attached to the effect of clumping on the coliform counts obtained in the previous experiment.

The marked bacteriostatic action of fresh "raw" milk on certain organisms is destroyed by boiling. Consequently there was a possibility that in the first experiment the rates of multiplication of the various coliform organisms might have been different if fresh unheated milk had been used instead of milk heated to 100°C. to effect sterilisation. The experiment was therefore repeated with fresh raw milk. A cow proved

to be free from mastitis was selected from which to obtain milk of a low bacterial content. The cow's udder and teats and the worker's hands were carefully washed with antiseptic. A considerable quantity of milk was discarded from each teat, then part of the remainder drawn directly into large sterile bottles. The milk was cooled immediately by immersing the bottles in cold water; thereafter brought to the laboratory and placed in a refrigerator. Then four hours after milking, the specimen was thoroughly mixed and distributed in 40 ml. amounts in sterile glass-stoppered bottles. The different lots of milk were then inoculated, as in the first experiment, from standardised suspensions of pure cultures of coliform bacteria. In this case, however, owing to the fact that other organisms might be present in the milk, a heavier inoculation was made to ensure that the coliform organisms would preponderate. Thus each bottle of milk was inoculated with 1 ml. of the 1/10,000 dilution of the primary suspension of the organism instead of 1 ml. of the 1/100,000 dilution. Twenty-nine cultures of different coliform organisms were used in this experiment, thirteen of these cultures were of B. coli types and sixteen of aerogenes-cloacae. As control, two bottles of milk were not inoculated.

The bacterial numbers present in the inoculated milks and in the controls were then determined by the plating method, triplicate plates being made with 0.1 ml. and 0.01 ml. quantities of the inoculated milks and with 1 ml., 0.1 ml. and 0.01 ml. quantities of the controls. The counting was carried out after the plates had been incubated at 37°C. for 48 hours. The number of coliform organisms with which each bottle of milk had been inoculated was then calculated by subtracting the average bacterial content of the two controls (282 organisms per ml.) from the bacterial content of the inoculated milk.

The bottles of inoculated milk and of the controls were kept at 17° C. for 24 hours and then the number of organisms in each again determined. In this case plates were made with 0.001 ml., 0.0001 ml. and 0.00001 ml. quantities of the inoculated milks and 0.01 ml., 0.001 ml. and 0.0001 ml. quantities of the controls. The plates were incubated at 37°C. for 48 hours and the counts made. The final coliform count of each bottle of inoculated milk was then calculated by subtracting the average bacterial content of the two controls after incubation (2,800 organisms per ml.) from the final bacterial content of the inoculated milk.

The results of this experiment are given in Table 5, page 151. The rates of multiplication of twenty-three of the cultures had been determined in sterilised milk as well as raw milk and therefore a comparison could be made in the case of each of these cultures between the rates of multiplication in the sterilised and in the raw milk. See Table 6, page 152. It will be seen from Tables 5 and 6 that the raw milk had a marked bacteriostatic action on certain strains of both B. coli and aerogenes-cloacae types, the aerogenes-cloacae types being generally affected to a much less extent than the B. coli. (This is what one would expect, taking into account the fact that the aerogenes-cloacae types, unlike the B. coli, are frequently encapsulated). The rates of multiplication of the various organisms were on the whole much lower with the raw milk than with the sterilised. Nevertheless, in the case of the raw milk, as in the case of the sterilised, the aerogenes-cloacae types multiplied as a rule much more rapidly than the B. coli. Thus the findings of the first experiment, i.e., aerogenes-cloacae types multiply much more rapidly than B. coli in milk held at 17°C., hold good no matter whether raw or heat-sterilised milk is used.

In carrying out the experiment with raw milk, the inoculated specimens after 72 hours' incubation at 17°C. were examined microscopically by the procedure already described, to determine whether there was a tendency for the organisms to occur in clusters. It was found that even after this prolonged period of incubation there was, with the exception of three strains of organisms, little or no tendency to clumping, the cells occurring for the most part either singly or in pairs. Clusters were occasionally observed, but these consisted in many instances of cocci derived from the original flora of the milk. Breed and Stocking (1920) obtained similar results with samples of raw milk inoculated with coliform organisms.

The investigation was then continued to determine whether similar results to those of the foregoing experiments would be obtained if mixed cultures, each consisting of a B. coli type and an aerogenes-cloacae type, were grown at low temperatures in milk.

2. <u>The rate of multiplication of B. coli, B. cloacae, B. oxytocus and B. aerogenes types, when growing in mixed cultures of coliform types in milk held at 17°C.</u>

The mixed culture in all instances consisted of a B. coli and an aerogenes-cloacae type. As in the

previous experiments, saline suspensions of pure cultures of the organisms were prepared and standardised by the opacity method to contain approximately 300,000,000 organisms per ml. Then 1/100,000 dilutions of the suspensions were made in sterile water, and the mixed culture was prepared by adding 0.5 ml. of the B. coli suspension and 0.5 ml. of the aerogenes-cloacae suspension to 50 ml. of sterile milk. The bacterial content of the milk was now estimated, the number of each type of organism present being determined by a method, which was based on the fact that cultures of aerogenes-cloacae types in many instances do not grow at 45°C. (See "The growth of B. coli and aerogenes-cloacae types at 37°C. and 45°C.", page 91). Only those aerogenes-cloacae cultures were used which failed to grow at this temperature. The procedure adopted was briefly as follows:- dilutions of 1/10 and 1/100 were prepared from the milk, and plates were made in quadruplicate for 1 ml. 1/10 ml. and 1/100 ml. amounts of the milk. Of each set of quadruplicate plates, two were incubated at 37°C. and two at 45°C. The colonies were counted at the end of 48 hours' incubation. The number of colonies on the plates which had been incubated at 45°C. indicated the number of B. coli organisms present in the milk, and the number of colonies on the plates incubated

at 37°C. indicated the number of organisms of both B.coli and aerogenes-cloacae types. By subtracting the first count from the second, the number of aerogenes-cloacae organisms present in the milk was obtained. The ratio of the number of bacteria of aerogenes-cloacae type to the number of B. coli type was now estimated.

The inoculated milk was incubated at 17°C. for 24 hours, and then a second count was made of each type of organism present, the procedure being the same as before, but dilutions of 1/100, 1/1,000, 1/10,000 and 1/100,000 being employed. The ratio of the number of aerogenes-cloacae organisms to the number of B. coli was estimated. Then by comparing this ratio with the corresponding ratio for the initial counts, the relative rates of multiplication of the two types of organisms present in the milk were determined.

The results of this experiment are shown in Table 7, page 153, only the ratio expressing the rate of multiplication of the aerogenes-cloacae type relative to the B. coli type being given. It will be seen from the table that with forty mixed cultures of an aerogenes-cloacae with a B. coli type, the former in thirty-four instances multiplied more rapidly than the latter, while in only six instances did the reverse occur. However,

there were great variations; thus the ratios expressing the relative rates of multiplication of the aerogenes-cloacae and B. coli types ranged from 90.7 to 0.1 although the majority (twenty-eight out of forty) were between 25.6 and 2.8.

3. The influence of temperature on the relative rates of multiplication of cultures of B. coli and aerogenes-cloacae types, when growing in mixed cultures of coliform types in milk.

It was evident from the results obtained both in the second and in the first part of this investigation that the aerogenes-cloacae types of bacteria generally multiplied much more rapidly in milk at 17°C. than did the B. coli types. While the second part of the investigation was in progress, the question arose whether temperature had any marked influence on the results. Accordingly, in twenty-one instances the milk, after being inoculated with the cultures of the two types of coliform bacteria, and the plates for the estimation of the bacterial numbers had been made, was divided into four portions. These were incubated for 24 hours respectively at 17°C., 22°C., 30°C. and 37°C. The number of B. coli and the number of aerogenes-cloacae in each portion of the milk were then de-

termined by the method already described; and the ratio of the aerogenes-cloacae count to the B. coli and the ratio expressing the rate of multiplication of the aerogenes-cloacae to that of the B. coli were estimated as shown on page 77.

The results of this experiment are given in Table 8, page 154, only the ratios expressing the relative rates of multiplication of the aerogenes-cloacae and B. coli types at the different temperatures being shown. It will be seen that at 17°C. the aerogenes-cloacae types multiplied more rapidly than the B. coli in sixteen instances out of twenty; at 22°C., they did so in eighteen cases out of twenty-one; at 30°C., in only five instances out of twenty; and at 37°C., in only four out of twenty-one. Accordingly temperature has a marked effect on the relative rates of multiplication of the aerogenes-cloacae and B. coli types in milk. There may be considerable variation between different cultures of the same type with regard to the rate of multiplication at a given temperature, but the aerogenes-cloacae types generally multiply more rapidly than the B. coli in milk held at 17°C. and 22°C., while the B. coli types in most instances multiply more rapidly than the aerogenes-cloacae in milk held at 30°C. and 37°C.

4. <u>The coliform flora of raw milk, which has been inoculated with bovine faeces, and held for 36 hours at 17°C.</u>

This experiment was carried out to determine whether enrichment of aerogenes-cloacae strains occurs when bovine faeces containing them are inoculated into milk and the culture kept for 36 hours at 17°C. The milk used in the experiment was unheated fresh milk of the Scottish "Certified" grade from farms known to produce clean milk of low bacterial count. On its arrival in the laboratory it was subjected to the coliform test, 1 ml. quantities being inoculated in triplicate into MacConkey's bouillon. Although the results of this test could not be determined until the bouillon cultures had been incubated for two days, the milk was used immediately for experimental purposes, the results being discarded when the milk was shown to contain coliform bacteria.

In carrying out the experiment, the milk was added in 50 ml. amounts to sterile bottles. The bottles were then inoculated with different specimens of bovine faeces, which were from one to three days old. In the case of the first six faecal specimens two loopfuls of the faeces were used as the inoculum in each instance. But with the last six specimens the quantity of the

inoculum was reduced and various amounts were used. Thus the practice was to prepare a fairly heavy suspension of each of these faecal specimens in sterile water and to inoculate separate bottles of milk with 1 ml., 0.5 ml. and 0.25 ml. of the suspension. The bottles of inoculated milk were incubated in all instances at 17°C. for 36 hours. Then three plates of MacConkey's agar were stroked in series from each bottle and incubated at 37°C. for two days. Over thirty colonies were picked off each set of triplicate plates and transferred to MacConkey's bouillon. Accordingly over thirty bouillon cultures were obtained from each bottle of inoculated milk, i.e., over thirty bouillon cultures from each of the first six faecal specimens and over ninety from each of the last six specimens. The bouillon cultures which produced acid and gas were replated in MacConkey's agar to ensure their purity. Then a sub-culture in MacConkey's bouillon was made from each plate. If acid and gas were produced the organisms were examined microscopically, after staining by Gram's method, and identified by their Voges-Proskauer, Koser, inositol and indole reactions. See Section IV, page 93.

The results of this experiment are given in Table 9, page 155. It will be seen that in fourteen instances out of twenty-four, strains of the aerogenes-

cloacae and intermediate types together constituted the greater proportion of the flora of the milk. In twelve cases the aerogenes-cloacae strains by themselves were in excess of the B. coli, while in other twelve cases the reverse occurred. With four of the six faecal specimens when 0.25 ml. of the faecal suspension was used as the inoculum, aerogenes-cloacae strains were obtained from the milk, and in three of these instances they were the predominant coliform strains. Accordingly, although aerogenes-cloacae types occur in bovine faeces only in comparatively small numbers, they become enriched in milk inoculated with the faeces and held at 17°C. Consequently they are frequently the predominant coliform types in such milk.

DISCUSSION

The significance of these results with regard to the incidence of various coliform types in unpasteurised market milk requires to be considered and in this connection the following facts are of importance.

1. Previous work (Malcolm 1933) has shown that the most prevalent types of coliform bacteria in unpasteurised market milk are B. coli communior and B. coli communis Escherich and these, according to the results of the

previous part of the present investigation, are also the most prevalent coliform types in bovine faeces. Further, there is a very high correlation between the relative numbers of B. coli communior and B. coli communis in milk and in bovine faeces, B. coli communior occurring in each case with greater frequency than B. coli communis. On the other hand, these coliform types have been found by numerous investigators to be of comparatively rare occurrence on fodder, grain and in soil in the absence of recent faecal contamination.

2. It has also been shown (Malcolm 1933) that the relative frequency of strains of different coliform sub-groups in unpasteurised market milk varies widely according to winter and summer conditions. During winter 71.0 per cent. of the strains obtained from specimens of such milk were typical B. coli, 16.1 per cent. were aerogenes-cloacae types and 12.9 per cent. were intermediate types, whereas during summer 40.4 per cent. of the strains were typical B. coli, 32.2 per cent. were aerogenes-cloacae types and 27.4 per cent. were intermediate types.

3. In winter there is a much greater possibility of gross faecal contamination of the milk than in

summer. This is due to the fact that in Scotland during winter the cows are confined almost entirely to byres, being allowed out during the day to the fields only for a brief period for exercise. In summer they are at pasture and are in the byres only at milking time.

4. It has been found in the present investigation (a) that aerogenes-cloacae types as a rule multiply much more rapidly in milk at low temperatures, 17°C. to 22°C., than B. coli types, this being the case whether raw or partially sterilised milk is used, and (b) that the coliform flora of milk which has been inoculated with bovine faeces and held for 36 hours at 17°C. frequently consists chiefly of aerogenes-cloacae types. These results are in agreement with those of other investigators. Thus, Rogers, Clark and Lubs (1918) quote Ayers as finding that when sterile milk was inoculated with bovine faeces and incubated at 20°C. the aerogenes types predominated at the time of curdling, and they found that this also holds good for human faeces. Hammer (1928) states that the "Aerobacter type" can grow in milk at a lower temperature than the "Escherichia type", so that it is of more importance from the standpoint of development in milk under

ordinary conditions.[+] From these facts the inference may be drawn that if milk which is contaminated with faecal matter is kept at temperatures of from 17°C. to 22°C., it may ultimately contain large numbers of aerogenes-cloacae types, even though these organisms occurred scantily in the faeces. Such a state of affairs is liable to arise in summer, because as previously mentioned 17°C. (63°F.) is a common temperature for farm milk at that period. On the other hand, milk is generally kept in winter at such low temperatures, e.g., 5°C. (41°F.) to 13°C. (55°F.), i.e., at about 10°C. (50°F.), that there is little or no growth and proliferation even of aerogenes-cloacae types. (Ayers and Clemmer (1918) and Finkelstein (1919) found that there was little or no growth of any coliform types in milk at or below 10°C.) Consequently the relative incidence of the different coliform types in the milk will tend to remain unaltered, and will correspond fairly closely with that of the different types in the faeces with which it was contaminated.

5. B. lactis aerogenes and B. oxytocus, i.e., the

[+]Wilson (1935) found that when eight mixed cultures of B. coli and B. aerogenes strains were grown for 24 hours in milk at 22°C., the B. coli in all instances multiplied more rapidly than the B. aerogenes. But he makes no claim that general conclusions can be drawn from his experiments owing to the small number of strains examined.

encapsulated types of coliform bacteria, are much more resistant to adverse conditions generally than the non-capsulated types, e.g., the typical B. coli. Thus Clemesha (1912), Rogers (1918), Winslow and Cohen (1918), Gray (1932), Burke-Gaffney (1933), Platt (1935) and others, have found that B. aerogenes has greater powers of resistance and can generally survive longer than B. coli in water. Accordingly, if milk utensils which have become contaminated with coliform organisms as a result of faecal contamination of the milk or of the utensils themselves, are not properly washed and sterilised, the B. aerogenes types will very possibly survive in them to a greater extent than the B. coli types. This is supported by the results of an investigation carried out by the late Professor R.H.Leitch of The West of Scotland Agricultural College. In a personal communication he stated that he found that milk is frequently infected with coliform bacteria and especially aerogenes types from the utensils.

In view of these facts and also those, discussed in pages 12 to 15, with regard to the possibility of milk being contaminated with aerogenes-cloacae types from non-faecal sources, e.g., fodder, bedding, cereal grains and soil, it seems reasonable to suppose that not

only the typical B. coli, but also the aerogenes-cloacae types, present in milk are derived to a considerable extent from bovine faeces. The relatively higher incidence of the latter in summer milk can be explained in terms of the foregoing findings as being due to a combination of factors. Their greater hardiness will allow them to survive in improperly sterilised utensils, while the temperature of the environment in the summer months will be almost optimal for their multiplication both in the utensils, e.g., the cans, strainer and cooler, and in the milk itself. Thus given faecal contamination of the milk, for which there is abundant evidence, even the small numbers of aerogenes types present in the faeces will under these suitable conditions for growth form a large proportion of the coliform types in milk during the summer months. They will be, however, relatively rare in winter when conditions are more suitable for gross faecal contamination of the milk, and when owing to the low temperature of the environment there will be comparatively little growth even of these types of coliform organisms in the utensils and in the milk.

SUMMARY

1. Seventy-five pure cultures of various types of coliform bacteria were grown in sterilised or partially sterilised milk for 24 hours at 17°C. The cultures of B. aerogenes, B. oxytocus and B. cloacae types as a rule multiplied much more rapidly than those of the B. coli types. Similar results were obtained when twenty-nine cultures of various coliform types were grown in raw milk for 24 hours at 17°C. There was generally little or no tendency to clumping of the organisms in the milk, even after 3 days' incubation at 17°C.

2. Forty mixed cultures, each consisting of an aerogenes-cloacae type and a B. coli, were grown in milk for 24 hours at 17°C. In thirty-four instances the aerogenes-cloacae type multiplied more rapidly than the B. coli.

3. Twenty-one of these mixed cultures were also grown in milk for 24 hours at 22°C., 30°C. and 37°C. At 22°C., as at 17°C., the aerogenes-cloacae type multiplied as a rule more rapidly than the B. coli, while at 30°C. and 37°C. the reverse was the case.

4. Twelve specimens of bovine faeces were inocul-

ated into raw milk and the cultures kept at 17°C. for 36 hours. Aerogenes-cloacae types occurring in the faeces became enriched in the milk, the coliform flora of the milk at the end of the incubation period frequently consisting chiefly of these types.

5. The greater incidence of aerogenes-cloacae types in summer milk in Scotland as compared with winter milk, found in a previous investigation, may be explained by the fact that when milk is contaminated directly or indirectly with faeces, these types become enriched at the temperatures of holding commonly employed in summer. Such enrichment does not occur in winter, the holding temperature of the milk being as a rule so low that there is little or no proliferation of any coliform types. Consequently the relative proportions of the various coliform types in winter milk tend to remain similar to those in the faeces.

NOTE TO SECTION III.

The growth of B. coli and aerogenes-cloacae types at 37°C. and 45°C.

All the cultures of B. coli type used in Section III. of the investigation grew well in bouillon at 45°C. There was, however, a possibility that the agar plate counts of such cultures might be less, if the plates were incubated at 45°C. than at 37°C. An experiment was therefore carried out to determine whether this was the case. Bouillon cultures of nine different B. coli strains, which had been sub-cultured daily for several days, were plated out on yeastrel milk agar. Six parallel plates were made for each of the following quantities of the cultures:- 0.00001 ml., 0.000001 and 0.0000001 ml. The plates were incubated for two days, three of each set of parallel plates being kept at 37°C. and the remaining three at 45°C. The counts were then made. The following table shows the bacterial content per ml. of each culture as determined by plating at 37°C. and at 45°C.

Strain	Bacterial content per ml. of bouillon culture when plates incubated at - 37°C.	45°C.
1.	270,000,000	280,000,000
2.	160,000,000	150,000,000
3.	300,000,000	420,000,000
4.	205,000,000	300,000,000
5.	140,000,000	150,000,000
6.	269,000,000	423,000,000
7.	390,000,000	380,000,000
8.	410,000,000	360,000,000
9.	570,000,000	450,000,000

It will be seen from the table that in most instances there was a close correspondence between the counts for each strain. Accordingly with B. coli strains the accuracy of the plate count is not affected by employing an incubation temperature of 45°C. instead of 37°C. On the other hand, it was found that fourteen out of fifty-five aerogenes-cloacae strains grew in bouillon at 45°C. Consequently, this method of making a differential count of B. coli and aerogenes-cloacae types can only be employed provided such atypical aerogenes-cloacae strains are absent. The fourteen aerogenes-cloacae strains which grew at 45°C. were therefore not used in investigating the relative rates of growth of B. coli and aerogenes-cloacae types in milk.

S E C T I O N I V.

THE CLASSIFICATION OF COLIFORM BACTERIA

The coliform organisms comprise a large group of lactose-fermenting, Gram-negative, rod-shaped bacteria. 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.

In the investigations of the coliform flora of milk (Malcolm 1933) and bovine faeces (see Section II, page 41), 1636 cultures of lactose-fermenting coliform bacteria were isolated, 797 from milk and 839 from bovine 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 this 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 in that and the present investigation. 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 sub-cultures 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 sub-groups, each of which is characterized by an assemblage of outstanding features. The sub-groups 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 sub-groups are intermediate in character. These four sub-groups 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 sub-divide the coliform group into eight sub-groups, each consisting of a number of MacConkey and other types (see Table 10, page 156).

The biological characters of the sub-groups.

Sub-group 1 (B.coli types): Voges-Proskauer-negative, Koser-negative, inositol-negative, indole-positive. The organisms of this sub-group 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 10);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 sub-group.

The types belonging to this sub-group 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 11, page 158). They are also the most prevalent forms in milk (see Table 11), 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 10) 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.

Sub-group 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 sub-group. Hay (1932) also reports the occurrence of such Koser-positive 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 sub-group.

It will be seen from Table 10 that only eighteen cultures of this sub-group 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 (see Section II, page 41). It

would therefore appear that such types are of comparatively rare occurrence in faeces and in milk. This sub-group 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 sub-group, after subculturing for several years in artificial media gave Koser-negative reactions, in other words they were similar to the typical B. coli.

Sub-group 3: Voges-Proskauer-negative, Koser-positive, inositol-negative and indole-negative. This sub-group 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 10).

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 sub-group. 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 sub-group or sub-group 5.

Altogether 126 cultures of sub-group 3 were ob-

tained 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.

Sub-group 4: Voges-Proskauer-negative, Koser-positive, inositol-positive and indole-positive. This sub-group 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 sub-group 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 sub-group 4 resemble the

B. coli sub-group 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 sub-group 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 sub-group produced trimethylene glycol and included these types in the genus Citrobacter.

Only twenty-three cultures of types of sub-group 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 sub-group 4 are of comparatively rare occurrence in milk, faeces, soil and water.

Sub-group 5. (B. friedländeri types): Voges-Proskauer-negative, Koser-positive, inositol-positive

and indole-negative. This sub-group 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 sub-group 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 naso-pharynx may give the biological reactions of typical B. aerogenes.

Sub-group 6. (B. cloacae types): Voges-Proskauer-

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

positive, Koser-positive, inositol-negative and indole-negative. This sub-group includes MacConkey's types nos. 6, 69, 73, 75,[1] 105 and 108, the prototype being no.108 (B.cloacae or Aerobacter cloacae). The organisms of this sub-group 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.

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

Of the total number of cultures isolated (see Table 10), 211 belonged to this sub-group; 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 11). It would therefore appear 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 sub-group cannot therefore be regarded as non-faecal types.

Sub-group 7. (B. oxytocus types): Voges-Proskauer-positive, Koser-positive, inositol-positive and indole-positive. This sub-group 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

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

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 10, that with few exceptions the cultures of this sub-group fermented adonitol, sucrose, dulcitol and even inulin. No other sub-group contained so high a proportion of inulin-fermenters. The majority of the cultures also slowly liquefied gelatin. Seventy-one cultures of this sub-group 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).

Sub-group 8. (B. aerogenes types): Voges-Proskauer-positive, Koser-positive, inositol-positive and indole-negative. This sub-group 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 sub-group 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 10 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 sub-group 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 10. 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 used as sub-group criteria, sub-groups 1, 2, 3 and 6 consisting of types which are negative either in inositol or adonitol, and sub-groups 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 sub-group 7, and eleven cultures in sub-group 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 sub-group 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 sub-group, 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 sub-groups. Twenty of these cultures apparently belonged to the Koser-positive sub-groups, 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 sub-groups. 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 sub-groups.

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 sub-group 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 sub-groups, the number of such cultures obtained is comparatively small, amounting in these investigations of milk and bovine faeces 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 sub-groups 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 (sub-group 1) are particularly well-defined owing to the correlation which exists between the sub-group 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 sub-group 1, the typical B. coli; the Voges-Proskauer-negative Koser-positive cultures belonged to sub-groups 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., sub-group 1 (or typical B. coli), failed to ferment inositol. Voges-Proskauer-negative inositol-positive types belonged to sub-groups 4 and 5. As regards the Voges-Proskauer-positive cultures, 291 (chiefly of B. aerogenes and B.

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 10.

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 (sub-groups 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 sub-group 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 sub-group 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 sub-groups 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 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 sub-group 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 cul-

tures belonged chiefly to sub-group 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 10). However, cultures of subgroups 4, 5, 6, 7 and 8 (i.e., all the inositol-positive sub-groups and also the B. cloacae), with the exception of only one culture, fermented sucrose; and all the cultures of sub-groups 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 sub-groups 2-8, in most instances (560 out of 583) gave positive reactions, while Koser-negative types, i.e., types of sub-group 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

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

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.

As previously mentioned, though coliform bacteria have certain characters in common, they differ widely from each other with regard to various other characters, the true systematic value of which is unknown. This constitutes a great difficulty in the classification of these organisms. 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 significance 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 significance, 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 (1916a, 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, sub-groups 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, sub-groups 6, 7, and 8 (the genus Aerobacter 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 sub-group 1 (B. coli types), and Koser-positive in the case of sub-groups 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 (sub-groups 2, 3, 4 and 5) are placed in the same genus, Escherichia, as the Voges-Proskauer-negative,

Koser-negative types (sub-group 1 or B. coli types). However, types of sub-groups 4 and 5 in many respects resemble very closely sub-group 8, the B. aerogenes types. Thus they are not only Koser-positive (and in the case of sub-group 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, sub-group 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) and Bardsley (1934). Its employment for systematic purposes

enables sub-group 1 or B. coli (Koser-negative types) to be differentiated from the other sub-groups (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 (sub-groups 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 the antiseptic action of brilliant green than Koser-negative (See Section II, page 41). 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) and Hay (1932). In the case of Voges-Proskauer-negative types, it sharply-differentiates sub-groups 1 (B. coli), 2 and 3, from sub-groups 4 and 5 (B. friedländeri); and in the case of Voges-Proskauer-positive types, it differentiates sub-group 6 (B. cloacae) from sub-groups 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 (sub-groups 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 (sub-groups 1, 2, 3 and 6) are as a rule motile and have not so high fermentative powers as the inositol-fermenters. Also, except for sub-group 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., sub-group 1 or B. coli types, fail to ferment inositol. This rule applies even to the types of this sub-group, 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., sub-group 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 sub-group 3, and many belonging to sub-group 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) and others have stressed the importance of the indole reaction for the differentiation of coliform types and sub-groups. Its employment, along with the other three reactions, for systematic purposes enables sub-group 1, the B. coli, to be very well-defined - a most important matter in public health work. Further, it enables sub-group 2 to be distinguished from sub-group 3; and sub-group 4 from sub-

group 5. It also allows sub-group 7 (B.oxytocus) to be differentiated from sub-group 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.)

While indole-negative types are Koser-positive, indole-positive types vary in their Koser reactions, the non-inositol-fermenters being Koser-negative and the inositol-fermenters, Koser-positive. There are very few exceptions to this rule and these belong chiefly to sub-group 2. Thus the B. cloacae types (sub-group 6) and B. aerogenes types (sub-group 8) are indole-negative and Koser-positive; the typical B. coli (sub-group 1) are indole-positive, Koser-negative and inositol-negative; and the B. oxytocus types (sub-group 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 sub-groups, because these reactions do not correlate highly with the Voges-Proskauer, Koser, inositol and indole reactions. It is,

however, worthy of note that sub-groups 4, 5, 7 and 8, i.e., all the inositol-positive sub-groups, and also sub-group 6, the B. cloacae, are sucrose-positive, while sub-groups 1, 2, and 3 are inulin-negative. The adonitol reaction correlates almost completely with the inositol with the exception of adonitol-positive types of sub-group 1 (the B. coli sub-group). These types are similar in other characters to other types of this sub-group, 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 sub-group, are inositol-negative, even although they give positive adonitol reactions. The mannitol reaction is of no value for differentiating sub-groups or types as all lactose-fermenting coliform bacteria ferment this substance. The salicin reaction is also of no value as a sub-group criterion, as types of sub-groups 2-8 (the Koser-positive types) as a rule give positive reactions while types of sub-group 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 sub-groups 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 sub-groups 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 (sub-group 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 sub-group. 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 that the coliform group should not be divided into two or more genera. The inclusion of the numerous types in one genus is justified by the close inter-relation shown by them in their biological characters and in their relations to environment. The group, however, may be divided into eight sub-groups, by employing the Voges-Proskauer, Koser, inositol and indole reactions as sub-group criteria. Of these sub-groups, 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 sub-groups are intermediate in their characters between the B. coli and the B. aerogenes; thus the non-inositol-fermenting Voges-Proskauer-negative types, i.e., sub-groups 2 and 3, resemble fairly closely the B. coli;

the non-inositol-fermenting Voges-Proskauer-positive types, i.e., sub-group 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., sub-groups 4, 5, and 7, are very similar to the B. aerogenes.

(2) Of all the coliform sub-groups no.1 (the B. coli or typical B. coli) is widely recognised 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 11). 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 sub-groups, 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 (sub-groups 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 sub-group 1 (B. coli types) in water is evidence of recent sewage pollution. On the other hand, if organisms of only sub-groups 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 (sub-groups 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, for systematic purposes, the group may be subdivided into sub-groups, 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 encap-

sulated and form thick, mucoid colonies. Non-inositol-fermenters are as a rule motile and with the exception of a few typical B. coli, 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 the following well defined sub-groups:-

Sub-group 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.

T A B L E S

TABLE 1. Cultures of coliform bacteria found in bovine faeces by the ordinary methods of isolation, (a) and (b), during the winter period (when cows were confined to byres) and during the summer period (when cows were at pasture.)

Sub-group of types.	BIOCHEMICAL REACTIONS				NUMBER OF CULTURES ISOLATED		
	Voges Proskauer-	Koser	Inositol	Indole	Winter	Summer	Whole period
* 1. B. coli	-	-	-	+	211	119	330
3. Intermediate	-	+	-	-	2	0	2
5. B. friedländeri	-	+	+	-	1	0	1
6. B. cloacae	+	+	-	-	2	0	2
7. B. oxytocus	+	+	+	+	3	0	3
8. B. aerogenes	+	+	+	-	4	0	4
					223	119	342

* Numbered to correspond with Tables 3 and 10.

TABLE 2. Types of Koser-positive coliform bacteria found in bovine faeces by enrichment methods of isolation, (a), (b), and (c), during the winter period (when cows were confined to byres) and during the summer period (when cows were at pasture).

Sub-group of types.	BIOCHEMICAL REACTIONS				NUMBER OF CULTURES ISOLATED		
	Voges-Proskauer	Koser	Inositol	indole	Summer	Winter	Whole period
* 2. Intermediate.	-	+	-	+	6	5	11
3. Intermediate	-	+	-	-	3	26	29
4. Intermediate	-	+	+	+	5	6	11
5. B.friedländeri	-	+	+	-	7	2	9
6. B.cloacae	+	+	-	-	39	50	89 ø
7. B.oxytocus	+	+	+	+	14	4	18
8. B.aerogenes	+	+	+	-	54	32	86
					128	125	253

* Numbered to correspond with Tables 3 and 10

ø Six cultures gave indole-positive reactions.

TABLE 3. Showing biochemical reactions and motility of 595 cultures of coliform bacteria isolated by ordinary and enrichment methods from bovine faeces.

Sub-group of types.	Voges-Proskauer	Koser	Inositol	Indole	Motility +	Motility -	Adonitol +	Adonitol -	Sucrose +	Sucrose -	Dulcitol +	Dulcitol -	Inulin +	Inulin -
1. B.coli (330 cultures)	-	-	-	+	292	38	30	300	210	120	281	49	0	330
2. Intermediate (11 cultures)	-	+	-	+	7	4	3	8	8	3	8	3	0	11
3. Intermediate (31 cultures)	-	+	-	-	30	1	0	31	16	15	5	26	0	31
4. Intermediate (11 cultures)	-	+	+	+	1	10	11	0	11	0	5	6	1	10
5. B.friedländeri (10 cultures)	-	+	+	-	2	8	10	0	10	0	4	6	1	9
6. B. cloacae (91 cultures)	+	+	-	-	82	9	3	88	91	0	33	58	6	85
7. B. oxytocus (21 cultures)	+	+	+	+	2	19	20	1	21	0	18	3	15	6
8. B. aerogenes (90 cultures)	+	+	+	-	10	80	85	5	90	0	21	69	19	71

TABLE 4. The rate of multiplication of different types of coliform organisms in pure culture in sterilised or partially sterilised milk held at 17°C. for 24 hours. The cultures belonging to each sub-group are arranged in ascending values of the ratios of final counts to initial counts.

Type of organisms	Initial count per ml.	Final count per ml.	Ratio of final count to initial count.
B. coli sub-group. (30 strains).	3,700	70,000	19
	4,800	90,000	19
	79,600	1,960,000	25
	13,900	500,000	36
	14,900	650,000	44
	4,400	200,000	45
	39 +	2,300	59
	18,500	1,110,000	60
	59 +	4,300	73
	13,700	1,020,000	74
	33 +	2,600	79
	6,800	570,000	84
	35 +	3,100	89
	1,660	160,000	96
	12,800	1,240,000	97
	11,600	1,150,000	99
	7,000	740,000	106
	41 +	4,900	120
	29 +	3,500	121
	43 +	5,400	126
	59 +	8,100	137
	6,600	1,090,000	166
	6,900	1,170,000	170
	29 +	5,900	203
	3,800	770,000	203
	30 +	7,900	263
	33 +	9,000	273
	88 +	25,600	291
	28 +	8,500	304
	49 +	20,900	427
B. cloacae sub-group (11 strains)	2,400	1,555,000	648
	100 +	66,000	660
	50 +	37,900	758
	36 +	29,000	806
	66 +	54,000	818
	3,050	2,970,000	974
	56 +	63,000	1,125
	61 +	71,000	1,164
	46 +	95,000	2,065
	1,050	3,530,000	3,362
	7,600	47,610,000	6,264

TABLE 4. (Contd)

Type of organisms.	Initial count per ml.	Final count per ml.	Ratio of final count to initial count.
B. oxytocus sub-group (20 strains)	14 +	11,600	829
	58 +	52,300	902
	19 +	19,000	1,000
	1,400	1,470,000	1,050
	40 +	44,000	1,100
	47 +	53,000	1,128
	131 +	161,000	1,229
	58 +	72,000	1,241
	33 +	42,000	1,273
	77 +	105,700	1,373
	36 +	50,000	1,389
	60 +	86,000	1,433
	47 +	89,000	1,894
	55 +	105,000	1,909
	74 +	168,500	2,277
	81 +	205,500	2,537
	850	3,150,000	3,706
	57 +	215,000	3,772
	450	2,105,000	4,678
	800	6,880,000	8,600
B. aerogenes sub-group (14 strains)	3,700	2,135,000	577
	56 +	35,300	630
	7,900	6,130,000	776
	18 +	15,400	856
	119 +	129,000	1,084
	4,250	4,665,000	1,090
	43 +	67,000	1,558
	47 +	99,000	2,106
	65 +	140,000	2,154
	1,400	3,390,000	2,421
	46 +	145,000	3,152
	7,200	23,240,000	3,228
	8,300	26,830,000	3,233
	3,800	14,840,000	3,905

+ Employing standardised suspensions. See text.

TABLE 5. The rate of multiplication of different types of coliform organisms in pure culture in a specimen of raw milk held at 17°C. for 24 hours. The cultures belonging to each sub-group are arranged in ascending values of the ratios of final counts to initial counts.

Type of organisms	Initial count per ml.	Final count per ml.	Ratio of final count to initial count
B.coli sub-group (13 strains)	1,868	700	0.5
	2,618	1,200	0.5
	2,208	2,700	1
	2,638	10,200	4
	2,908	16,200	6
	3,518	22,700	6
	6,118	34,200	6
	4,118	51,200	12
	1,328	58,700	44
	3,418	171,700	50
	2,318	122,200	53
	3,018	198,200	66
	2,218	169,200	76
B.cloacae sub-group (3 strains)	4,018	80,200	20
	2,198	502,200	228
	1,958	3,997,200	2,042
B. oxytocus sub-group (7 strains)	10,218	18,200	2
	2,418	827,200	342
	2,618	992,200	379
	2,318	2,052,200	885
	2,148	2,762,200	1,286
	2,338	3,794,200	1,623
	2,118	4,197,200	1,982
B. aerogenes sub-group (6 strains)	4,218	4,700	1
	1,788	627,200	351
	2,818	2,237,200	794
	3,448	3,137,200	910
	2,818	4,997,200	1,773
	3,118	7,597,200	2,437
Control without addition of any culture.	282	2,800	10

The initial and final counts of each coliform type were obtained by subtracting the initial and final counts respectively of the control from the initial and final counts of each milk culture.

TABLE 6. The rate of multiplication of different types of coliform organisms in pure culture in (a) sterilised milk and (b) raw milk, held at 17°C. for 24 hours. The rate of multiplication is expressed as the ratio of final count to initial count of the milk.

	RATE OF MULTIPLICATION	
	Sterilised Milk	Raw Milk
B. coli (10 strains)	25	6
	44	50
	99	53
	120	4
	137	12
	166	6
	170	0.5
	203	0.5
	291	6
	427	66
Aerogenes-cloacae (13 strains)	578	1
	630	910
	660	228
	776	351
	902	379
	1,084	794
	1,229	1,623
	1,373	342
	1,433	885
	2,154	1,773
	2,277	1,286
	2,537	1,982
	3,938	2

TABLE 7. The relative rates of multiplication of aerogenes-cloacae types to B. coli, when growing in mixed culture in milk at 17°C. for 24 hours.

Experiment.	Ratio of multiplication rates: aerogenes-cloacae to B. coli.	Experiment.	Ratio of multiplication rates: aerogenes-cloacae to B. coli.
1.	5.3	21.	0.6
2.	31.8	22.	11.0
3.	6.3	23.	3.7
4.	33.4	24.	2.0
5.	1.4	25.	12.5
6.	0.4	26.	5.2
7.	19.4	27.	3.0
8.	8.3	28.	9.0
9.	7.8	29.	0.3
10.	5.0	30.	25.6
11.	24.4	31.	14.1
12.	7.7	32.	3.1
13.	11.0	33.	6.3
14.	52.5	34.	4.0
15.	5.0	35.	0.1
16.	4.9	36.	0.3
17.	2.8	37.	14.1
18.	0.2	38.	4.0
19.	90.7	39.	21.2
20.	7.4	40.	17.4

TABLE 8. The influence of temperature on the relative rates of multiplication of aerogenes-cloacae and B. coli types, when growing in mixed culture in milk for 24 hours. Each figure indicates the ratio of the multiplication rate of the aerogenes-cloacae type to the multiplication rate of the B. coli.

Experiment	TEMPERATURE OF INCUBATION			
	17°C.	22°C.	30°C.	37°C.
21.	0.6	9.2	0.9	5.0
22.	11.0	13.3	-	11.7
23.	3.7	43.1	0.3	0.4
24.	2.0	77.0	1.7	1.3
25.	12.5	7.0	0.3	0.2
26.	5.2	20.7	1.3	0.3
27.	3.0	1.5	0.3	0.3
28.	9.0	34.8	0.2	0.1
29.	0.3	0.2	0.7	0.1
30.	25.6	21.5	1.3	0.4
31.	14.1	23.1	0.1	0.3
32.	3.1	1.5	3.8	0.3
33.	6.3	5.0	0.3	0.7
34.	4.0	7.4	0.5	0.2
35.	0.1	0.7	0.2	0.1
36.	0.3	0.4	0.1	0.4
37.	14.1	8.9	0.1	0.3
38.	4.0	5.4	0.2	0.4
39.	21.2	19.8	0.3	0.4
40.	17.4	25.5	0.2	0.1
41.	-	46.7	7.2	3.8

TABLE 9. The coliform flora of milk which has been inoculated with bovine faeces and held for 36 hours at 17°C.

Faecal Specimen	NUMBER OF CULTURES ISOLATED FROM THE MILK		
	B. coli types	Intermediate types.	Aerogenes-cloacae type (B.aerogenes, B.cloacae B.oxytocus and B. friedlanderi).
1.	-	-	26
2.	1	5	23
3.	6	4	3
4.	1	3	-
5.	-	-	2
6.	-	2	7
7. (a.	28	-	-
7. (b.	14	-	-
7. (c.	10	1	-
8. (a.	33	-	-
8. (b.	30	-	1
8. (c.	30	2	-
9. (a.	-	5	9
9. (b.	2	3	10
9. (c.	6	1	18
10. (a.	26	-	-x
10. (b.	7	-	-
10. (c.	8	-	-x
11. (a.	-	1	13
11. (b.	4	1	6
11. (c.	2	-	8
12. (a.	5	3	1
12. (b.	2	-	7
12. (c.	4	-	12

x Cultures of aerogenes-cloacae types were obtained on re-examination of colonies on original plates.

Inoculum consisted in "a" of 1 ml. of faecal suspension; in "b" of 0.5 ml. and in "c" of 0.25 ml.

TABLE 10. Showing biological characters of 1597 cultures of coliform bacteria obtained from milk and bovine faeces by ordinary and by enrichment methods of isolation.

Sub-groups of types.	$CO_2:H_2$	Voges-Proskauer	Koser	Inositol	Indole	MacConkey's types
1. B.coli	Low	-	-	-	+	1,2,4,5,33, 34, 35, 71, 72, 100, 106, 107.
2. Intermediate	"	-	+	-	+	As above but Koser-positive.
3. Intermediate	"	-	+	-	-	7,36,70,74, 109.
4. Intermediate	"	-	+	+	+	66,[1] 101.
5. B.friedländeri	"	-	+	+	-	68,99,104[1]
6. B.cloacae	High	+	+	-	-	3,69,73,75[1] 105,108
7. B.oxytocus	"	+	+	+	+	65,97[1]
8. B.aerogenes	"	+	+	+	-	67,98,102, 103.

(The mannitol, raffinose, salicin and methyl-red reactions have

1. Differing from MacConkey's type in inositol reactions. Thirty-

not included in the table. Details of their characters are given

Motility		Adonitol		Sucrose		Dulcitol		Inulin		Gelatin		Total
+	-	+	-	+	-	+	-	+	-	+	-	
785	97	106	776	512	370	692	190	0	882	5	877	882
13	5	7	11	12	6	12	6	0	18	1	17	18
124	2	1	125	48	78	40	86	0	126	9	117	126
1	22	23	0	23	0	7	16	1	22	0	23	23
4	29	33	0	33	0	14	19	5	28	3	30	33
198	13	4	207	211	0	59	152	13	198	141	70	211
4	67	70	1	71	0	55	16	59	12	47	24	71
34	199	222	11	232	1	42	191	43	190	24	209	233

not been included for reasons given in text.)

nine cultures out of a total of 1636 were of atypical strains & are

in text.

TABLE 11. Types of coliform bacteria isolated.

Sub-groups of types	Voges-Proskauer	Koser	Inositol	Indole	By ordinary methods		By enrichment methods.
					Milk	Bovine faeces.	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.

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