The bacteriological examination of water supplies.

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THE BACTERIOLOGICAL EXAMINATION OF WATER SUPPLIES.

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PREFATORY NOTE BY THE CHIEF MEDICAL OFFICER.

To the Right Hon. Sir E. Hilton Young, G.B.E., D.S.O., M.P., Minister of Health.

Sir,

This Memorandum on the Bacteriological Examination of Water Supplies is the work of a small Office Committee which included, in addition to members of the staff of the Department, several distinguished authorities whose help I wish here gratefully to acknowledge.* Special tribute is due to the late Sir Alexander Houston, whose wide knowledge and lifelong experience were placed freely at the disposal of the Committee up to a few weeks before his death.

In Public Health Bacteriology of recent years in England there has been insufficient uniformity in the technique employed by different workers in the examination of water supplies, and, in consequence, neither the actual data obtained nor their hygienic interpretations have been comparable. The present Memorandum describes the technical steps commonly used in sound practice which can be relied upon to yield a reasonably complete picture of the bacterial content of water, so far as it may affect the health of consumers. It is not to be regarded as a rigid specification the non-observance of which would invalidate the conclusions drawn from an examination of a sample of water. The bacteriologist has a wide discretion in the range and thoroughness of the tests he may apply to any sample. He may be led to simplify his procedure by knowledge already in his possession of the quality of the particular water under examination, by the degree of importance of the deductions expected, or even by the expense involved in carrying out a full examination. He may, for example, use a smaller number of plates in the colony count; he may modify the "presumptive coli" test, or, as is sufficiently indicated in the text, he may refrain from a more minute investigation of the bacteria responsible for a "presumptive coli reaction." At some future date it may be possible to specify different grades of bacteriological test applicable to various contingencies.

Neither is the technique herein described to be regarded as a new contribution to the science of bacteriological analysis of water.

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No novel procedure is recommended, nor is any new body of observations brought forward. Indeed, several procedures already proposed or used by workers at home and abroad have not been mentioned, since, as yet, they cannot be regarded as part of generally accepted doctrine. Furthermore, the standards of purity, in terms of bacteriological control, suggested in Section VI. of the Memorandum, are put forward with due reserve. It may not be found practicable to attain them with some water supplies which, nevertheless, may justly be passed as satisfactory. Or it may be found that the limits suggested as permissible should be made more stringent and that water supplies just coming within those limits should be subjected to further processes of purification till they reach a higher degree of quality.

Special attention is drawn to the importance of correlating exact topographical conditions with bacteriological findings, and to the fallacy, not indeed current among bacteriologists themselves, of drawing final conclusions from a single bacteriological examination of any water.

In brief, the Memorandum may be regarded as correlating tests of which many modifications are at present current, and as suggesting standards for a routine in water examinations, thus contributing to a greater degree of unanimity of opinion on the hygienic quality of any of our diverse water supplies.

I have the honour to be, Sir,

Your obedient Servant,

GEORGE NEWMAN.

Whitehall,
March, 1934.

THE BACTERIOLOGICAL EXAMINATION OF WATER SUPPLIES.

Preliminary Note.

This Memorandum has been prepared for circulation with the following objects :—

(1) To provide a description of technique the adoption of which will ensure sufficient uniformity in the practice of bacteriological examination of water to permit of comparison of the results obtained in different laboratories.

(2) To explain to Sanitary Inspectors and others the precautions necessary in obtaining and transmitting samples to the laboratory.

(3) To assist in assessing the results of bacteriological examination in terms of hygienic quality.

I. INTRODUCTION.

The bacterial flora of different waters varies so much that it is impossible to lay down absolute standards to which all safe waters must conform. Each supply must be judged on the sum of knowledge obtained by topographical and laboratory examination in the light of accumulated hygienic experience of water supplies in general. Even from the same supply, a sample of water may, on a particular arbitrary laboratory standard, give one day a result indicating potential danger. Later, perhaps the next day, a similar examination may give a result apparently suggestive of safety; yet from the epidemiological point of view the supply has probably not altered at all. On the other hand, frequent and regularly repeated examinations, which reveal the normal range of variation of the flora, often enable a standard to be established for any one water so that any marked variation from such a norm would at once be regarded with suspicion.

The main object of the bacteriological examination of water is to find whether excretal pollution is present, but a single examination, however favourable the result, does not justify the conclusion that the water is suitable for drinking purposes. Not until several examinations with satisfactory results have been made at different times of the year, and especially after heavy rainfall, is it legitimate to conclude that the water is free from dangerous excretal pollution. Reasons may exist for suspecting that a water supply may be liable to pollution in circumstances that only occur at long intervals. For example, a prolonged summer drought, with an increased seasonal demand for water, (often augmented by an increased summer population), may involve the drawing of underground supplies from a much wider area than normal, with consequent risk of polluted infiltration reaching the water-bearing strata. Especially when such a drought is followed by heavy

rains, there is the risk of polluted surface water gaining access to the supply, whether it is a deep well, a spring or a river. At such seasons frequently repeated bacteriological examinations are called for in order that, if necessary, appropriate treatment may be applied. Not only is it desirable to insist on frequent examination of a water supply, but it is also important to realise that the most which bacteriological tests can prove is that *at the time of examination* bacteria indicative of excremental pollution did or did not under laboratory conditions grow from a sample of the water. Even a satisfactory result, apart from other data, affords no assurance that at some future time dangerous pollution will not occur. For this reason the results of bacteriological examination should always be reported with due caution and interpreted in conjunction with the known topography.

Normally the bacteriological examination of water is not directed to the search for specific pathogenic organisms, which are difficult to isolate and usually signify no more than has already appeared by the occurrence of disease among the consumers. The evidence generally sought is (1) an estimate of the number of bacteria of all kinds capable of developing in suitable nutrient media—the greater the number, the greater presumably is the amount of decomposable organic matter present in the water : and (2) the number of bacteria of faecal origin ; the more bacteria, of species inhabiting normally the animal intestine, that are present in the water, the more likely is it that pathogenic intestinal species may gain access to it. The evidence is therefore circumstantial and, accordingly, often open to doubt in its interpretation.

II. TAKING OF SAMPLES.

The importance of good sampling cannot be over-estimated. Upon it depend the deductions which are ultimately drawn from the results of the examination. Every care must therefore be used to prevent any risk of contamination of the water in taking the sample.

Since the laboratory examination of a sample should always be considered in conjunction with the topographical circumstances of the supply, it is important in sending a sample of water for bacteriological examination, to send with it very full details of the nature and source of the supply and of the reasons that have led to a desire to have the water examined. Detailed instructions, setting out fully the precautions to be taken and the information that should be supplied, are given in Appendix A.

III. ROUTINE EXAMINATION FOR INDICES OF POLLUTION.

The examinations recommended are :—

(*a*) *Agar* count at 20°–22° C.

(*b*) *Agar* count at 37° C.

(*c*) *Coli-aerogenes* count.

Less often examination is made for the following organisms :—

(*d*) *Faecal Streptococci.*

(*e*) *Clostridium welchii.*

(*f*) Specific pathogenic organisms, especially *Bacterium typhosum* and *paratyphosum.*

(*a*) The *Agar count at* 20°–22° C is to be preferred to the gelatin count at the same temperature. Agar, while giving a somewhat similar count (often higher), is free from the disadvantage of possible liquefaction.* The use of agar also avoids a needless multiplication of media. Most bacteria developing at 20°–22° C. and not at 37° C. are non-pathogenic to human beings, and it might therefore be thought that their number was immaterial. On the other hand, their number gives some indication of (1) the amount of food substance available for bacterial nutrition and (2) the amount of soil, dust, and other extraneous material that has gained access to the water. For example, after heavy rain the 20°–22° C. count will sometimes show a striking rise, not paralleled by the 37° count.

(*b*) *The Agar count at* 37° C. affords more information as to dangerous pollution. Most of the natural harmless water bacteria do not grow readily at 37° C. ; the organisms developing at this temperature are chiefly those of soil, sewage, or intestinal origin, and their number, therefore, may be used as an index of the degree of purity of the water (*vide* p. 20).

(*c*) *The coli-aerogenes count.*

The coli-aerogenes group is generally held to include all Gram-negative non-sporing rods capable of fermenting lactose with the production of acid and gas, and of growing aerobically on agar media containing 0·5 *per cent. bile salt.*

Since the number of such organisms in water is likely to be comparatively small, the count is made not in a solid medium but by cultivating measured quantities of the water sample in a suitable liquid nutrient broth such as that of MacConkey, *vide infra.* It is assumed that the implantation of a single living bacillus of this group into such a medium is generally followed by growth which alters the character of the medium and is rendered evident by the use of indicators for acid and gas production. The calculation is made accordingly that with a graded series of inoculations, the smallest quantity of water producing this change contains one such bacillus. The method suffers from the disadvantage that, if organisms of both the coli and the aerogenes types are inoculated into the same tube, one or other may gain the ascendancy, with the result that, when the culture is subsequently plated out, colonies of only one species may be detected. Nevertheless, it has proved its worth

* There is no special significance in gelatin-liquefying organisms as such.

as an index of excretal pollution of water and it may safely be said that, just as the 37° count is of greater significance than that af 20°–22°, so is the " coli-reaction " a more faithful indication of faecal pollution than the number of organisms growing at 37° C. Moreover, experience has shown that it is easier to lay down absolute standards for the coli-aerogenes test than for either the agar count at 37° or that at 20°–22°.

The work of the past few years has made it clear that, among the organisms producing the coli-aerogenes reaction in MacConkey broth (acid and gas), a distinction can be made between the typical faecal *Bact. coli* on the one hand and the *Bact. aerogenes* on the other, and that the presence of the former in water must be regarded with greater suspicion than that of the latter, which is commonly present in cultivated soil and on vegetation. Since, however, there are a number of intermediate types, and since even aerogenes may be found in small numbers in the intestinal canal (having gained access to it presumably with food), it is impossible to assess the real importance of the presence of this bacterium, and especially of the intermediate types, as indicative of excretal pollution. It is necessary, too, to remember that the bacterial flora of sewage and, in even greater degree, that of sewage-polluted water differs from that of human faeces in the relative proportion of the species of the coli-aerogenes group. But sewage and sewage-polluted waters are none the less potentially dangerous to health if they gain access to drinking water. And it should not be forgotten that the presence of the less " suspicious " organism, aerogenes, though perhaps merely the result of access of soil washings, may convey a warning, since experience has shown the sinister association of flood water and water-borne disease. The differentiation, however, remains desirable, and the interpretation that can be placed on the finding of aerogenes instead of coli is likely to become more definite as experience accumulates. Deductions already fairly well established are that (1) the presence of faecal coli is indicative of recent excretal pollution, since the majority of faecal coli die out within two or three weeks ; and (2) the presence of aerogenes in the absence of faecal coli is much less reliable evidence of such pollution. Under certain conditions aerogenes may survive in polluted water long after the faecal coli introduced by the same pollution have disappeared, and thus may be a significant finding. On the other hand, aerogenes may have been introduced with soil, vegetable matter, or dust, so that too much importance should not be attached to its presence. In the routine examination of water, a preliminary estimation of the numbers of coli-aerogenes bacteria is, therefore, made on the basis of acid and gas production in MacConkey broth, and a subsequent differentiation of the species producing the reaction may follow, entailing a number of tests such as the indole, methyl-red, Voges-Proskauer and citrate, *vide infra*.

(*d*) *Faecal streptococci content.* Streptococci of various types are present in a number of situations in the human body. In the faeces the most characteristic type is that known as *Str. faecalis. This organism is a Gram-positive coccus, generally occurring in pairs or short chains, producing no change in raffinose, producing acid but not gas in mannitol, and being more resistant to heat than most vegetative bacteria.* It is generally regarded as non-hæmolytic, though on blood-agar plates it may sometimes give rise to a slight degree of α-haemolysis. In searching for it, advantage is taken of its ability to grow in MacConkey's broth, and of its heat resistance. Its presence is indicative of contamination of the water with faecal matter. Though not usually looked for, it is of particular value when the results yielded by the coli-aerogenes test are anomalous. When, for example, it is doubtful whether a certain atypical coli, which may be present in large numbers in the water, is or is not of faecal origin, the accompanying presence of *Str. faecalis* then affords valuable information as to the probability of the coli being derived from faeces. In normal faeces, faecal streptococci may be present in nearly as great numbers as *Bact. coli*, and similar standards of interpretation are therefore applicable in relation to the numbers present.

(*e*) *Cl. welchii content.* In bacteriological work on water and sewage this organism was formerly referred to as *B. enteritidis sporogenes.* By improved anaerobic technique it has been found that stock cultures of *B. enteritidis sporogenes* were, in fact, mixtures of two organisms—*Cl. welchii*, a natural inhabitant of the intestinal canal, and *Cl. sporogenes*, a natural inhabitant of sewage. *Cl. welchii is a stout Gram-positive rod, forming elliptical subterminal spores considerably wider than the bacillus itself, though spores are not usually formed in laboratory media. One of the most characteristic properties of this organism is the production of a change in litmus milk generally referred to as "stormy fermentation."* The casein of the milk is coagulated as the result of a rennet-like ferment ; the lactose is broken down with the production of acid and gas ; the gas blows the clot to pieces, and the litmus is reduced, and hence decolorized, by the growth of the organisms. The presence of *Cl. welchii* in water may be detected by either of two methods : (1) by seeding suitable quantities of freshly boiled litmus milk with the original water, heating the mixture to 80° C. for 10 minutes to destroy non-sporing organisms, incubating anaerobically for three days at 37° C. and observing the development of stormy fermentation ; and (2) by applying the sulphite-reduction test. This test was introduced in 1925 by W. J. Wilson, of Belfast, as an indication of faecal pollution of water, additional to that obtained by the coli-reaction, and depends on the fact that *Cl. welchii* produces conspicuous black colonies when grown in agar containing glucose, sodium sulphite and iron.

(f) The search for specific pathogenic organisms.

In a water containing relatively few micro-organisms the search for specific pathogenic bacteria such as *Bact. typhosum* or *paratyphosum* is generally fruitless, even though there may be strong evidence to suggest that the water has been responsible for the production of enteric fever. The reason for this is partly that the organisms are present in only small numbers or may have actually disappeared at the time of examination, and partly that until recent years no reliable selective medium has been available for their detection. If a given water receives a single contamination, from a typhoid carrier for example, it will probably be about a fortnight before a case of typhoid fever develops, and perhaps another week before the case is diagnosed and reported to the Medical Officer of Health. If the contamination has not been repeated, it is therefore probable that the typhoid bacilli will have died out, so that a search for their presence is foredoomed to failure. If, on the other hand, the contamination has been repeated, and especially if, as when a leaky water pipe admits sewage, the contamination has been continuous, the chance of finding the bacilli is rather greater. Since in water containing typhoid bacilli there are always a large number of coli present as well, the difficulty in the past has been to prevent the overgrowth of the typhoid by the colon bacilli. The introduction of Wilson and Blair's medium* has been a considerable step forward in surmounting this difficulty, for not only is the growth of coli suppressed on this medium but also the typhoid colonies take on a characteristic appearance making them easily recognisable. For this reason water that is under suspicion of conveying enteric fever should be examined for the presence of organisms of the typhoid-paratyphoid group. Since, however, the search for and identification of these organisms are difficult, and demand considerable experience, the examination can only be undertaken in well equipped and adequately staffed laboratories.

IV. TECHNIQUE OF BACTERIOLOGICAL ANALYSIS OF WATER.

In the following pages the various procedures to be carried out in a "standard" bacteriological examination are described in considerable detail. The purpose of this detailed description is not necessarily to prescribe a rigid set of rules from which any deviation is to be deprecated, but rather to call attention to the numerous points in technique which may affect the final observations. It is left to the individual worker to decide whether deviation at any point will result in increased convenience or cheapness without appreciable loss in accuracy or in comparability with the tests of

* Vide *J. of Hyg.*, 1931, **31**, 138.

other workers. In many cases considerable modification will be justified in order that the results of examination of a particular water supply may be comparable with a long previous series, and where daily or weekly bacteriological control of a single supply is practised, a much simplified set of tests may often be adequate for the purpose. Comments on the technique herein described and suggestions for its amendment, arising out of experience of its use, will be welcome and should be addressed to the Medical Department, Ministry of Health, Whitehall, London, S.W.1.

The necessary apparatus and its preparation are described in detail in Appendix B, and recipes and methods for the preparation of media in Appendix C. These prescriptions should be strictly followed if uniform results are to be obtained. For the same reason the use of certain proprietary preparations of established merit has occasionally been prescribed.

The examination should be commenced immediately after receipt of the sample at the laboratory. If this is impracticable, the sample should be placed in the ice-chest until it can be examined. Not more than eight hours should elapse between the collection of the sample and its examination ; if this interval is longer, it should be expressly noted on the report.

(*a*) *Diluent.*

Tap water may be employed if it has been shown that it is free from any marked germicidal activity. Distilled water, if it has been distilled in glass *and not in metallic stills*, is also permissible. Preferably, one-quarter strength Ringer's solution should be used (*vide* Appendix C, XIX.).

(*b*) *Filling the dilution bottles with diluent.*

The sterile diluent should be measured out with aseptic precautions into sterile dilution bottles, either by a sterile 90 ml. delivery pipette or by a suitable sterile automatic all-glass delivery burette. Alternatively, sterile test tubes may be preferred instead of dilution bottles, and quantities of 9 ml. instead of 90 ml. of diluent put up. The loss in accuracy of dilution is not great and is balanced by the diminished cost of glass-ware.

(*c*) *Preparation of dilutions and inoculation of double strength MacConkey broths.*

The sample bottle should be inverted 25 times by a rapid rotatory movement of the wrist in order to distribute any deposit uniformly throughout the water. After flaming the mouth of the bottle, one quarter of its contents should then be poured off, the stopper re-inserted, and the bottle shaken 25 times, each shake being an up and down movement with an excursion of about one foot, the whole shaking to last 12 seconds. One, two, or, rarely, three dilutions, according to the nature of the sample, each ten times the previous one, should be made in dilution bottles containing 90 ml. of diluent, by carrying over 10 ml. of the water to the first bottle, and after suitable mixing carrying over 10 ml. of the 1 in 10 water to the second bottle, and so on. In the actual process of making the dilutions, a sterile 10 ml. pipette should be inserted into the sample bottle with its tip reaching not more than $\frac{1}{2}$ to 1 inch below the surface of the water. The water should be sucked up and down ten times approximately to the 10 ml. mark, in order to wet the interior of the pipette thoroughly ; 10 ml. of water should then be measured, holding the pipette in the vertical position, the lowest part of the meniscus just touching the 10 ml. mark. The pipette should be removed

from the bottle, its tip being touched during the process against the neck of the bottle about ½ inch below the rim, so as to remove any excess adhering to the outside. It should then be introduced into the first dilution bottle with the tip below the neck of the bottle but above the level of the diluent. The pipette should be held vertically and the water allowed to flow out by gravity ; before withdrawal, the tip of the pipette should be touched against the neck of the bottle at the lowest possible point. On no account must the pipette be allowed to come into contact with the diluent. With the same pipette five lots of 10 ml. of the original water should be transferred to tubes of double strength MacConkey medium. In the process the pipette should again be allowed to discharge by gravity, and before removal the tip should be touched against the side of the test tube about ½ inch above the level of the medium. The pipette should then be discarded. *Before sterilisation every pipette should be fitted with a plug of cotton-wool at the upper end to prevent contamination of the interior by organisms from the mouth.*

If it is proposed to take a 50 ml. quantity of the water for the coli count, this may now be done, using a 50 ml. bulb pipette to transfer it from the original sample bottle to a tube or bottle containing 50 ml. of double strength MacConkey broth.

The first dilution bottle should be shaken 25 times, a fresh 10 ml. pipette taken, and using exactly the same technique as before, 10 ml. of the 1 in 10 water should be transferred to the second dilution bottle.*

Alternatively, the dilutions may be made in test-tubes, 6 by ¾ ins., by transferring 1 ml. of the water to a test-tube containing 9 ml. of diluent, mixing thoroughly, and carrying over 1 ml. of the 1 in 10 dilution to a second test-tube containing 9 ml. of diluent. In the actual process of making the dilutions, a sterile 1 ml. pipette should be introduced into the sample bottle with its tip reaching not more than ½ to 1 in. below the surface of the water. The water should be sucked up and down ten times to the 1 ml. mark, and 1 ml. of water should then be measured out, holding the pipette in the vertical position. The pipette should be withdrawn, the tip being touched against the neck of the bottle to remove excess fluid adhering to the outside ; it is then introduced into the first test tube of the diluting series with the tip touching the side of the tube at a point about ½ in. above the level of the diluent. It is important that the pipette should not come into contact with the diluting fluid. The water should then be blown out, 3 seconds should be allowed to elapse for drainage, and the remaining contents blown out. The pipette should then be discarded. A fresh pipette should be introduced into the 1 in 10 dilution, with its tip reaching not more than ½ to 1 in. below the surface of the diluent, the fluid sucked up and down ten times to the 1 ml. mark, 1 ml. measured out with the pipette in the vertical position, the pipette removed from the fluid, its tip being touched against the side of the tube about ½ in. below the rim so as to remove any excess adhering to the outside, the pipette transferred to the second tube of the diluting series, introduced with its tip touching the side of the tube about ½ in. above the level of the diluent, and the contents expelled in the manner described above.

(*d*) *Inoculation of plates and of single strength MacConkey broths.*

A sterile 1 ml. delivery pipette should be taken and, commencing at the highest dilution and working up to the original water, 1 ml. portions should be transferred to sterile Petri dishes. In this process the pipette should be introduced into the second dilution (1 in 100 water) with its tip not more than ½ in. below the level of the fluid ; the fluid should be sucked up and down ten times, 1 ml. measured out, the pipette withdrawn, the tip touched against the interior of the neck of the bottle or test tube to remove excess fluid adhering to the outside, the 1 ml. blown out gently, so as to avoid

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* When the sample is known to be of a comparatively pure water, it is unnecessary to make the 1 in 100 dilution.

splashing, into the centre of a sterile Petri dish, the tip of the pipette being held about ½ in. above the level of the glass, an interval of 3 seconds allowed to elapse, the tip of the pipette touched against the glass at a point some distance from the fluid already delivered, and the last drop blown out. Two Petri dishes should be inoculated each with 1 ml. portions from the second (1 in 100) dilution,* four dishes from the first (1 in 10) dilution, and four dishes from the original water. At the same time as the Petri dishes are inoculated, five tubes of single strength MacConkey broth should be inoculated with 1 ml. portions from the first (1 in 10) dilution, if required, and five tubes from the original water, 10 tubes being inoculated in all. In the process of inoculation the pipette should be introduced into the test tube with the tip touching the side of the tube about ½ in. above the level of the broth ; the contents should be blown out, 3 seconds allowed to elapse, and the last drop blown out.

In the case of a water of which the normal bacterial content is known as the result of a long series of examinations, modifications of the procedure just described may be adopted, one dilution only, or even the undiluted water alone, being employed for inoculating plates, and the quantities chosen being such as to give an easily countable number of colonies and a suitable range in the MacConkey broth. For such waters also the bacteriologist may rightly prefer to continue examination with the technique responsible for previous results, so as to maintain historical continuity, reserving the technique of this Memorandum for unknown or less accurately known specimens.

(*e*) *Pouring of the plates.*

A sufficient number of tubes each containing 10 ml. of standard nutrient agar are boiled up, and cooled down to 50° C. Into each of four plates inoculated with the original water, each of four plates with the 1 in 10 water and, if required, each of the two plates with the 1 in 100 water, are poured 10 ml. of nutrient agar. Immediately the medium is delivered, mixing should be performed by a combination of rapid to and fro shaking and centrifugal movements lasting over a period of 5-10 seconds, the plate being kept flat on the bench throughout the whole process. The exact procedure consists in 5 to and fro movements followed by 5 centrifugal movements in a clockwise direction, succeeded by 5 to and fro movements at right angles to the first set followed by 5 centrifugal movements in an anti-clockwise direction. After mixing, the plates should be allowed to stand, if necessary on ice, for about one hour, after which they should be transferred to the incubator.

The time that elapses between the preparation of the dilutions and the pouring of the plates, should in no case exceed 15 minutes.

(f) *Incubation.*

Two of each set of four plates made with the original water and with the 1 in 10 dilution should be incubated at 37° C. for two days, and two at 20°–22° C. for three days, in each case bottom upwards. The two plates made with the 1 in 100 dilution should be incubated at 20°–22° C. for three days.

If water-jacketed incubators are not available, it is important to check frequently the temperature of the incubator in the

* See footnote on p. 10.

neighbourhood of the plates so as to make certain that it does not rise too high. In many electrically heated 37° C. incubators it is not uncommon for the air near the radiants to reach a temperature of 45° C. The atmosphere of the incubator should be kept moist. If stacking of the plates is necessary, they should be staggered in such a way as to interfere as little as possible with the circulation of air round them in the incubator; no stack should be more than six plates high unless a cellular incubator, in which the heating is from the sides, is used. All the tubes of MacConkey broth should be incubated at 37° C. for 48 hours.

(g) *Counting the plates.*

(i) Plates incubated at 37° C.: These should be removed from the incubator at the end of 48 hours.

Plates containing between 30 and 300 colonies should be selected for counting, the remainder being discarded. No plate containing less than 30 colonies should be counted unless the plates made from the undiluted water contain less than this number, and no plate containing more than 300 colonies should be counted (except when it is one of a pair of which the other gives a count of 300 or less) unless the plates made from the 1 in 10 dilution contain more than this number; in either of these events, the result must be returned as "approximate only." If there are more than 300 colonies in both plates made from the 1 in 10 dilution, the count may be reported as "more than 3,000 colonies per ml." If on naked eye inspection there appears to be a gross discrepancy between the numbers of colonies in plates made from different dilutions, a rider should be added in the report, drawing attention to this fact. Plates should be counted within four hours of their removal from the incubator; if this should be impracticable, they should be placed in the ice-chest till they can be counted. The best method of counting is with a specially constructed box allowing of examination of the plates by combined reflected and transmitted artificial light against a dark background. Direct daylight should be prevented from reaching the plate, by means of an opaque screen. The plate should be examined with a magnifying glass of 4 in. focal length giving a magnification of approximately 2½ diameters. Every object that is of such a size, shape, colour, opacity and refractivity as to leave no doubt in the observer's mind that it is really a colony, should be counted as a colony; small objects in the medium about whose nature there is any doubt should be disregarded.

(ii) Plates incubated at 20°–22° C.: These should be removed from the incubator at the end of 72 hours.

The same principles should govern the enumeration of colonies in these as in the 37° plates. If there are more than 300 colonies in the plates made from the 1 in 100 dilution, the plate count may be reported as "more than 30,000 colonies per ml."

(h) *Reporting the results.*

Water contains a large number of different species of organisms a certain proportion of which are dead. Under aerobic conditions of incubation in the standard nutrient media recommended, not all of the living organisms find conditions suitable for development. Since, moreover, some of the organisms occur in the form of pairs, groups, chains, or even dense clumps, not every living organism that is capable of developing gives rise to a separate colony. It is therefore clear that the number of colonies does not correspond with the total number of organisms present in the water. For this reason *the plate count results should be reported, not in terms of the number of organisms per ml., but as the number of colonies developing per ml. of the original water.* For estimating this figure, all plates containing between 30 and 300 colonies should be taken, the number of colonies multiplied by the reciprocal of the dilution, and the arithmetic mean determined.

Of the plates made with the undiluted water, it may be found that one of a pair incubated at the same temperature develops less than 30 colonies and the other more than 30, or that of the 1 in 10 dilution one of the plates develops more than 300 colonies and the other less. In either of these cases the mean of the two plates should be taken in estimating the number of colonies per ml. The same applies to plates of 1 in 100 dilution incubated at 20°–22° C.

If in both plates made from the original water the number of colonies is less than 30, the mean of the two plates should be taken, and the results expressed as "approximate only." If in both 37° plates (made from the 1 in 10 dilution), or in both 20°–22° plates (made from the 1 in 100 dilution), there are more than 300 colonies, the results should be expressed as "more than 3,000 colonies per ml.," or "more than 30,000 colonies per ml.," respectively.

(j) *Examination of the MacConkey broths.*

After 24 hours' incubation the MacConkey broths should be examined, and all tubes showing both acid and gas formation regarded as "presumptive positives." If it should be judged desirable to undertake confirmatory and differential tests, these tubes should at once be plated out (*vide infra*). The remaining tubes should be incubated for another 24 hours, and any further tubes that then become positive should be similarly regarded as "presumptive positives," and, if necessary, plated.

V. CONFIRMATORY AND DIFFERENTIAL TESTS.

Plating out.

In this country, MacConkey's neutral-red bile-salt lactose agar plates are ordinarily used. Other valuable media are, however, much employed abroad, as, for example, Endo-agar and Eosin-methylene-blue agar (Levine). It is claimed for some of these media

that they enable the observer to differentiate between *Bact. coli* and *Bact. aerogenes* by direct inspection of the colonies. With Eosin-methylene-blue agar, differences between the colonies of *Bact. coli* and *Bact. aerogenes* can, in fact, often be made out within 24 hours, though in 48 hours differentiation is much easier. The formulæ for the preparation of these media will be found in Appendix C and it is hoped that workers in this country who have the opportunity of doing so will employ them and report their results.

Petri dishes of standard size into which about 15 ml. of melted MacConkey agar have been poured *without bubbles* and allowed to set, are first well dried by several hours' incubation at 37° C. (or, preferably, by one hour in an incubator at 55° C), in the inverted position and with the inner dish which contains the medium supported on the edge of the cover ; such dried agar plates may be kept in stock, wrapped in paper, for several weeks in the refrigerator. A loopful (1 to 2 mm. diameter) of the primary culture, or preferably of a dilution of it prepared by mixing one loopful with one ml. of sterile broth, is then placed half way between the centre and the edge of the plate and spread over the surface by a sterile right-angled glass rod. In spreading, the drop should first be well distributed along the contact portion of the rod (about 1½ in. long) by a to and fro motion and the rod then drawn round the rest of the plate ; it should be kept gently but firmly in contact with the medium and parallel with the radii, until it has almost completed a circuit. Interruptions in the circuit by lifting the rod and re-applying a short distance further on help to increase the number of isolated colonies which is the object of plating ; the rod should not cover the same surface of agar more than once. Other devices for obtaining isolated colonies e.g., previous dilution of the primary culture, parallel and cross striations with loop or straight wire, may be used according to personal preference.

The plate is then incubated, bottom up, at 37° C. for 24 hours.

Investigation of colonies.

On MacConkey agar the coli-aerogenes colonies are characteristically red ; the depth of tint, however, varies considerably. The normal colony of *Bact. coli* is circular, convex, and not mucoid or brittle ; whereas the *Bact. aerogenes* colony is very often mucoid or viscous. For subculture and further test, one or more such colonies are selected from each plate, the number depending on the degree to which minor differences in colony appearance are perceptible and suggest the desirability of separate examination. Should no typical colonies appear on the plate within 48 hours, it is well to select at least one of the predominating variety for further investigation, bearing in mind the possibility that such atypical colonies may owe their different appearance (absence of redness) to a mere temporary slowing of their action on lactose.

In subculturing, a speck of the bacterial mass forming the colony is picked off with a wire or loop, emulsified in a tube of peptone water (formula VI) and incubated at 37° C. The colony subculture so prepared should, after 4 to 6 hours at 37° C., be used for the inoculation of 1 tube of peptone water for the indole test, 2 tubes of glucose phosphate medium (formula IX) for the methyl-red and Voges-Proskauer tests, 1 tube of citrate medium (formula XII) for test for growth, and 1 tube of gelatin (formula XI) for test for liquefaction. It is well to inoculate also a lactose and a sucrose peptone water tube.

Differential Tests.

The first clear distinction between the members of the coli-aerogenes group is to be found in the pioneer work of MacConkey (1900–1906). MacConkey defined the aerogenes type as being sucrose-positive, dulcite-negative, not producing indole, and reacting positively in the Voges-Proskauer test. These characters still form the basis of its differentiation with certain additions which we owe to American observers, (Rogers, Clark and Davis, Clark and Lubs, Koser).

In the following table, five tests are prescribed, by means of which seven common types of bacteria, all capable of producing the coli-aerogenes reaction in MacConkey broth, are differentiated by their varying behaviour. The reactions accredited to these bacteria are not absolutely constant and strains will be found possessing other combinations of them, though some such strains will, on more careful examination, be found to consist of a mixture of organisms. Moreover, the types can be further subdivided on the basis of other tests, such as motility, production of mucoid colonies, fermentation of sucrose, salicin, dulcitol, adonitol, inositol, etc., resulting, for example, in such varietal names as *Bact. coli-communius*, *coli-commune*, *acidi-lactici*, *neapolitanum*, etc. However valuable these distinctions may be from a scientific standpoint, there is no practical object in a water examination in differentiating between bacteria which may confidently be described as of faecal origin.

	Indole	V.P.	M.R.	Growth in citrate.	7 days gelatin lique-faction.
Bact. coli faecal type I ...	+	−	+	−	−
Bact. coli faecal type II ...	−	−	+	−	−
Intermediate type I ...	−	−	+	+	−
Intermediate type II ...	+	−	+	+	−
Bact. aerogenes type I ...	−	+	−	+	−
Bact. aerogenes type II ...	+	+	−	+	−
Bact. cloacae	±	+	−	+	+

The presence of faecal coli I and II in water is to be regarded as pointing strongly to recent excretal pollution. The members of the intermediate and aerogenes group and the cloacae type are commonly found in cultivated soil, vegetation, etc., so that their presence in water cannot be regarded as necessarily indicative of faecal contamination. These groups are apparently much more common in water supplies in the United States of America, in Canada, and in tropical countries generally, than in Great Britain, and their presence is regarded in those countries as of relatively small significance, since experience has shown that they are abundant in many waters otherwise hygienically satisfactory. In this

country, however, their presence is taken more seriously, since there is reason to believe that they are usually present, if only in small numbers, in faecal matter (having gained access to the intestinal canal in food), and since they are common inhabitants of sewage. Moreover, they may, in virtue of longer survival in water than faecal types, be the only indication of a heavy faecal pollution at some relatively distant previous date and warn us of its possible recurrence.

In the application of the five tests the following procedures are recommended :—

(1) *Indole test.*—The peptone water culture already referred to is removed from the incubator after 3 days and 1 ml. of ether added to it with vigorous shaking. It is then allowed to stand for a minute or two to allow of separation of the ether layer containing any dissolved indole and a few drops (0.5 ml.) of Ehrlich's reagent (*vide* Appendix C. XVII) allowed to run down the inside of the test tube. If the test is positive, the pink colour of rosindole appears within a few minutes.

(2) *Methyl-red Test.*—Glucose phosphate broth* is inoculated with a loopful of the stock peptone water culture 4–6 hours old, and incubated for 3 days at 37° C. Five drops of 0.04 per cent. methyl-red solution are then added to the culture. A magenta red colour is considered a positive result : a yellow colour is negative : pink or pale red are best considered as doubtful results.

(3) *Voges-Proskauer Test.*—Glucose phosphate broth similarly inoculated and incubated for 3 days at 37° C. has added to it 5 ml. of a 10 per cent. solution of sodium hydroxide. The result is read after the tube has stood 24 hours at room temperature, or after heating the mixture to near boiling point for about half an hour. If positive, a pink fluorescent colour develops : if negative, no colour.

A modified method has been put forward by O'Meara :—A knife point of creatin is introduced into the culture as above and 5 ml. of 40 per cent. potassium hydroxide added. The tube is shaken for 2 to 5 minutes. If positive, a pink colour develops : if negative, no colour.

(4) *Growth in Citrate.*—Tubes of citrate medium should be inoculated from the stock peptone water culture of 4 to 6 hours age by means of a straight wire, not a loop, so that the size of the inoculum will be roughly standardised and an opacity in the citrate tube due to size of inoculum and not to growth will be avoided. The tube is then incubated for 24 hours at 37° C., or, if no growth has occurred, for 48 hours. Opacity is considered evidence of growth.

(5) *Gelatin liquefaction.*—Inoculation of the solid medium should be performed with a straight needle charged with the stock peptone water culture (stab culture) or with a loop on the surface (slope culture) and the tube incubated at 20°–22° C. for 7 days.

Additional Tests.

These are largely confirmatory and may be of use in important cases, such as those of new supplies and where there is lack of agreement between topographical observations and bacteriological findings.

(6) *Eijkman's test.*—This depends on the observation that the faecal coli types grow at 45° C.,† whereas the aerogenes types fail to do so. The

* See Appendix C., IX.

† Experience has shown that 46° C., the temperature originally recommended by Eijkman, is too high.

inoculation is made in the same manner as in the citrate test, using the special medium (modified Eijkman, *vide* Appendix C. XVI) and incubating for 24 hours at 45° C. The production of gas is regarded as a positive reaction. It would appear, however, that some strains of faecal coli fail to grow under these conditions.

(7) *Motility.*—It is often stated that the majority of strains of faecal types of coli of human origin are motile, whereas *Bact. aerogenes* is often non-motile. A negative observation, however, is of doubtful value, since temporary loss of motility of *Bact. coli* is not uncommon. The observations may be made on "hanging drops" of the condensation water of moist agar slope cultures not more than 16 hours old, or on young broth cultures at 37° C.

(8) *Fermentation Tests.*—The fermentation of lactose is a useful confirmation of the "presumptive coli" reaction to which to subject the stock subcultures. The medium (Appendix C. II) is inoculated with a loopful, incubated at 37° C. and readings taken at 24 hours and subsequently as required. All the members of the coli-aerogenes group produce acid and gas, the great majority of strains in 24 hours.

The fermentation of sucrose, with abundant gas production in 24 hours, indicates that the strain most probably belongs to the aerogenes or intermediate group. Most strains of faecal coli either fail to ferment sucrose within 24 hours or they produce only a bubble of gas.

(9) *Gas ratios.*—The faecal types grown in dextrose broth produce a mixture of gases with carbon dioxide and hydrogen in about equal proportion, whereas the aerogenes types usually produce from 1.4 to 2.0 of carbon dioxide to 1 of hydrogen. The results of the test have been found to run parallel with those of the methyl-red test which is much more convenient.

Streptococcus Test.

This is generally employed as a confirmatory test only.

If lactose bile-salt cultures have been used for the primary cultures, it is easy and practicable to utilise them for the lactose+ faecal streptococcus test. It is not suggested that this test should be carried out in all cases, but it affords, in doubtful instances, confirmatory evidence of faecal pollution, and it may help to distinguish between contamination derived from man and that coming from animals, birds and other less reprehensible sources. It involves no delay in registering the final results, and, if carried out by the heating method (here described), is easy to perform and introduces practically no element of error. Its success depends on the circumstance that faecal streptococci resist heating operations to so much greater an extent than coliform organisms that, after heating, the original ratio between the two is completely altered. It is true, that in the heating process many streptococci perish, but this is largely got over by heating relatively huge amounts of the primary cultures and plating subsequently with amounts far in excess of what would be used in ordinary coli work.

After very thoroughly mixing the primary culture, 1 ml. (relatively speaking, an enormous amount) is withdrawn by means of a pipette and added to 9 ml. of sterile water in a test tube, the greatest care being taken to avoid side-smearing. The tube is immersed in a water-bath maintained at a temperature of 60° C., in which indeed it should have been standing

for some time previous to the inoculation. The depth of water in the bath should be considerably greater than the height of water in the test tube.

After ¼ hour* the tube is withdrawn, shaken, and by means of a sterile pipette, one drop (a relatively very large amount) is transferred to the surface of a MacConkey or agar plate, and the liquid spread over the medium by means of a sterile bent glass rod. The plate is incubated for 24 hours, at 37° C., and then examined. There may be no *Bact. coli*, or only a few, and the latter result is really to be preferred, as showing that the heating process has not been excessive. If streptococci were present in the primary culture, they will usually be found on the plate in considerable number and far in excess of any resistant *Bact. coli*. They appear as minute characteristic colonies. Several of them are picked off for subculture, each one being treated as follows :—

A colony is picked off with a sterile wire and a lactose peptone water tube inoculated ; the same wire, without going back to the original colony, is used to inoculate the condensation fluid of a nitrate agar† slope culture, the wire thereafter being drawn upwards and laterally to and fro. After 24 hours at 37° C., the tubes are examined. If the lactose tube shows either no change, or gas formation, this is regarded as a negative result. If, however, the lactose tube is acid without gas formation, and the contents towards the foot of the tube have a characteristic reddish floury appearance, the results will usually be found to be positive. The condensation liquid of the agar slope is next examined microscopically and a note made of the appearance of the colonies on the slope, which ought to be characteristically minute. Faecal streptococci are usually very short-chained and are easily recognisable. The great majority of bacteria isolated from water reduce nitrates to nitrites ; the streptococci do not possess this property. A few drops of metaphenylene-diamine solution‡ are added to the agar slope culture—no brown coloration should result.

The results should be recorded as the smallest amount of water yielding " lactose + morphologically satisfactory " streptococci.

Raffinose and mannitol tests :

If thought desirable, further cultures from the lactose tube may be made into raffinose and mannitol.

The *Streptococcus faecalis* of Andrewes gives negative results in raffinose, and positive in mannitol.

Nevertheless, there are many human faecal streptococci which do not conform to this rigid classification and these should certainly not be regarded as unimportant.

Clostridium welchii test.

(1) The " stormy fermentation " of milk may be used as an index of the presence of spores of this bacillus. Graded quantities of the water, say 100 ml., 20 ml., and 10 ml. are added to 100 ml. of sterile litmus milk in flasks, the mixtures heated at 80° C. for 10 to 15 minutes and incubated anaerobically. A positive reaction will appear after 24 to 72 hours at 37° C. ; a negative reaction is of little value in assessing the purity of water.

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* Shorter or longer periods may be tried, but ¼ hour is usually about right. Strictly speaking, the heating operations should not be pushed beyond the point of rendering the isolation of streptococci easy, where before it was impossible.

† See Appendix C, XV.

‡ See Appendix C, XVIII.

(2) The sulphite-reduction test. Two plates are made as described in Appendix C., V., and after 24-36 hours aerobic incubation at 37° C., the black colonies in the depth of the medium are counted.

These colonies usually fall into four types :—

(1) Large colonies, 5 m.m. or more in diameter. These are usually colonies of *Cl. welchii*, but not infrequently contain strains of the coli-aerogenes group.

(2) Medium-sized colonies, 3–4 m.m. in diameter. These also may be either *Cl. welchii* or coli.

(3) Small colonies, 1–3 m.m. in diameter.

(4) Tiny colonies less than 1 m.m. in diameter.

These types (3) and (4) are of less significance. Though absent from pure water, the bacteria producing them are probably saprophytic water bacteria, which may be present in a potable supply during the summer months.

The presence of large and medium colonies of types (1) and (2), indicates contamination, and Wilson regards the appearance of more than one large black colony in 40 ml. as showing the presence of *Cl. welchii* and justifying the suspicion of faecal contamination. His conclusion is based on the belief that *Cl. welchii* is not found in nature apart from faecal pollution: its power of producing resistant spores makes it a valuable index of intermittent contamination of water, in which circumstances the coli test may give a series of negative results.

The sulphite-reduction test of Wilson has not yet been widely reported upon publicly, but private reports are favourable, and it is worthy of a place in conjunction with the coli reaction. Recently Wilson (1931) has recommended incubation of his plates at 45° C., at which temperature *Cl. welchii* grows well whereas the other bacteria producing black colonies are restrained in growth, and this procedure is worthy of adoption in further trials. The test would appear to be of particular value in the examination of water from surface wells where contamination is very often intermittent, and hence not so easily detected by the ordinary procedures.

VI. INTERPRETATION OF RESULTS AND APPLICATION OF STANDARDS.

The interpretation of the results of bacteriological examination in terms of hygienic quality of water demands not only careful consideration of other relevant factors, but also a considerable experience. It is not the function solely of the Medical Officer of Health and the Sanitary Engineer to make this interpretation ; the bacteriologist, as a result of experience, necessarily acquires special knowledge of the significance of both major and minor observations, so that it is both his right and his duty to offer an opinion on the question in his report to the health authority. It cannot yet be said that final decisions have been reached as to the meaning of all the data obtainable in the laboratory ; conclusions are still to some extent reflections of individual experience and therefore variable. Hence it is not possible to lay down definite

numerical standards with which all waters should comply. The bacteriological condition of a water has to be considered in relation to many other factors such as season, nature and topography of the source of supply, the frequency of examination, etc., so that all that can be done here is to state some generally accepted deductions and to suggest broad lines for the hygienic classification of waters of similar origin. There are several admirable textbooks which will assist and fructify the bacteriologist's experience (Thresh, Beale and Suckling ; Savage ; Hewlett ; Horrocks, etc.), while the Annual Reports of the Director of Water Examinations of the Metropolitan Water Board not only form a corpus of valuable laboratory observations but also show what useful hygienic information can be derived from the routine examination of single supplies.

The *colony count*, as has already been indicated, is of value chiefly when carried out on the same supply at regular intervals over long periods. In such circumstances an increased count always requires explanation. It may depend merely on seasonal influences—in which case it will be paralleled by the results of previous years—but it *may* indicate undesirable access of storm water, of other polluted surface or soil water, or direct contamination by dust or animals. In the case of filtered water, not only should the counts furnish evidence of the efficacy of the filtration in terms of percentage removal of bacteria (95 to 98 per cent. with slow sand filters), but also a rise in the colony count is the usual signal of defect in the filter beds, demanding instant attention. The regular examination of filtered water at frequent intervals and with a technique adapted to secure economy and rapidity (e.g., with 2 plates only and readings at 24 hours) is, therefore, current practice.

The colony count of a single sample of water has, however, comparatively little significance in itself and it is difficult to state limits which, if exceeded, involve unfavourable comment on the hygienic quality of the water. Recently sunk wells and bores are likely for some time to give a misleading count. Bacterial contamination, necessarily introduced in the work of sinking, may persist for weeks or even months in a new well properly protected and drawing from an entirely unpolluted subsoil water. Such evidences of pollution should disappear when the well has " settled." In the case of " settled " artesian wells and deep springs, provided the water has not been long stagnant, the count on agar at 37° C. should not exceed 10 per ml. ; any excess would suggest admixture with water of more superficial origin or some other form of pollution. Surface wells, on the other hand, usually give counts higher than 10 per ml., but so many circumstances have to be considered in assessing the importance of the figure obtained that no standard can be laid down.

The ratio of the count at 22° C. to that at 37° C. is chiefly of use in helping to explain sudden fluctuations in the bacterial content of a water ; the higher the ratio, the more probable is it

that the bacteria are clean soil and water saprophytes and therefore of small significance. The ratio may depend somewhat on season and may tend to be low in summer and high in winter. In unpolluted water, the ratio of the 22° C. count to the 37° C. count is usually 10 or more to 1 ; in polluted waters it is usually below 10. But this rule is subject to so many exceptions that no weight can be attached to single observations. After chlorination of a supply the ratio has no significance : it is usually very low.

The *coli-aerogenes count*, or " presumptive coli " test, in the lactose bile-salt medium is, at present, the best bacteriological index of pollution of water. As already explained, it is based in a quantitative sense on the assumption that the smallest quantity of water giving the coli-reaction contained one viable bacillus of the group " coli-aerogenes," from which the total content in unit volume, say 100 ml., can be calculated, e.g., should the smallest quantity with a certain water be 10 ml. the calculation would be that there are 10 coli-aerogenes bacteria in 100 ml. Experience shows, however, that direct calculation in this way is fallacious, since parallel tests on the same sample of water may give different results, e.g., the smallest quantity giving a positive reaction may be 10 ml. in the first experiment, 50 ml. in a second, and 1 ml. in a third, whilst a fourth test may give a positive reaction in 1 ml. and a negative in 10 ml. This seemingly anomalous behaviour is due mainly to the fact that the test is one of random sampling, the positive and negative results depending on the chance distribution of viable coli-aerogenes bacteria in the water. It has frequently been the cause of differences, both in observation and in interpretation, between different analysts working on the same water. To obtain approximate accuracy in the numerical expression of the coli-aerogenes count one must, therefore, (1) put up a series of tests each with the same volume of the water, and (2) apply to the observations a formula based on the laws of probability. Such a formula has been devised by Greenwood and Yule.*

Appendix D gives two sets of figures expressing the numbers of bacteria of the coli-aerogenes group in 100 ml. of water, as shown by the various combinations of positives and negatives in one or other of the series of presumptive coli tests in MacConkey's lactose broth, proposed in this Memorandum. The figures have been computed by McCrady† by the use of Greenwood and Yule's formula, slightly modified.

If the series of tests employed is that in which one 50 ml. quantity, five 10 ml. and five 1 ml. quantities are put up, then Table I applies. If the series preferred is one with five 10 ml., five 1 ml. and five 0.1 ml. quantities then the probable number of the specific bacteria can be read from Table II. If one 50 ml. quantity, five 10 ml., five 1 ml. and five 0.1 ml. quantities have been put up, one or

* *J. Hyg.*, 1917, **16**, 36.

† *Canad. Pub. Health J.*, 1918, **9**, 201.

other of the two tables must be used according to the results obtained ; if all tubes seeded with 0.1 ml. are negative, then Table I applies ; if one or more tubes seeded with 0.1 ml. are positive then Table II should be used.

The following examples with three different waters will make this clear :—

Quantity of water put up in each tube.		50 ml.	10 ml.	1 ml.	0.1 ml.
No. of tubes put up ...		1	5	5	5
No. of tubes showing positive reaction :—	(*a*)	1	5	3	0
,, ,,	(*b*)	1	5	4	1
,, ,,	(*c*)	1	0	1	1
,, ,,	(c^1)	1	0	2	0

Combination (*a*) involves the use of Table I and gives accordingly the figure of 90 coli per 100 ml. Combination (*b*) requires Table II and gives the figure of 170 coli per 100 ml. When, as in example (*c*), a positive with 50 ml. is succeeded by a negative with the 10 ml. and one or more positives with 1 and 0.1 ml. quantities, the positive in the 0.1 ml. quantity is transferred to the 1 ml. results making the series read as in (c^1) and giving the figure 4 by Table I.

As has already been mentioned, bacteria giving the " presumptive coli " reaction may be either typical coli or aerogenes or intermediate species. In the light of present knowledge it is prudent to assume that " typical coli " are of faecal origin, and to accept the consequent implications. The true significance of the other two groups is still a matter of doubt, and further data as to their origin, habitat, mode of access to water and their longevity therein, are much needed, particularly as these may vary in different parts of the country. The Medical Department of the Ministry will be glad to receive from water bacteriologists any observations they may make in the future as the result of differentiating the " presumptive coli reaction " in the manner suggested in this Memorandum, especially in the case of water supplies giving moderate numbers of coli-aerogenes organisms. Such observations will be of great value in assessing the hygienic importance of the presence of the different bacterial species.

Experience so far acquired suggests that the majority of water bacteria producing the " presumptive coli reaction " are " true coli," and that no serious hardship to water suppliers in this country is involved in regarding the " presumptive coli reaction," without further differentiation, as an index of faecal contamination, unless this assumption is grossly discrepant with other information. The reaction is always a sign of undesirable possibilities.

It remains, however, to be considered whether there is any permissible number of " presumptive coli " such that a water

containing these organisms in this or smaller number may, without further differentiation of species, be regarded as suitable for domestic use. Ideally, " presumptive coli " should be absent from 100, 200 or more ml. of drinking water, but such an ideal standard would exclude many waters that can be and are consumed with impunity for indefinitely long periods ; so when the reaction gives, as the probable number, not more than 2 " presumptive coli " per 100 ml., the water may be regarded as reasonably satisfactory without further differentiation of these " presumptive coli."

On the other hand, when the " presumptive coli " reaction indicates that the water frequently contains these organisms in such numbers as (say) 10 or more per 100 ml. it may be assumed, without further differentiation of species, that the sources of supply are exposed to serious and possibly dangerous pollution, and a careful investigation of the conditions of the source should be made, the water meanwhile being regarded as unsuitable for domestic use.

An intermediate group of waters exists—those which give " presumptive " counts higher than 1 or 2 per 100 ml., yet not high enough to suggest that the conditions of the source are necessarily unsatisfactory. The " presumptive coli " in such waters may usefully be subjected to the differential tests described in this Memorandum. If, on such further examination, the " presumptive coli " are regularly found to be of the " aerogenes " or " intermediate " types, further steps towards greater purity of the source of supply are not immediately required, although, depending on the known conditions of origin (e.g., moorland water, deep wells, etc.), it will be wise to recommend somewhat frequent examination lest further deterioration in quality occur.

If a water known to have been originally polluted but subsequently purified by storage, filtration and/or chlorination, shows more than an occasional rise in the " presumptive coli " count, an investigation into the efficiency and working of the purification plant should be undertaken. Efficient chlorination should yield a water free from " presumptive coli " in 100 ml. Making allowance, however, for experimental error, whether in sampling or otherwise, a probable " presumptive coli " count of not more than 2 per 100 ml. may be allowed in 10 per cent. of the chlorinated samples without further question.

It should be emphasised that a single isolated examination of any water is rarely sufficient for a pronouncement on its hygienic quality unless it is so bad as to justify immediate condemnation. In " border-line " cases repeated examinations are always necessary, and the gravity of positive coli-aerogenes findings from a hygienic point of view is proportional to the frequency with which they appear in a series of samplings.

These remarks and suggested standards are applicable to large supplies where regular bacteriological testing is economically practicable. In the case of small supplies, and especially of shallow

wells such as are common in rural districts, decision is much less easy : the principles remain the same, but it may be impossible on economic grounds to adopt the suggested standards and subsequent treatment. Much may be done, of course, by local measures such as removal of obvious sources of pollution, to bring such waters closer to the standard, but, furthermore, the discovery that the " presumptive coli " reaction (frequently given by them) was due to the " aerogenes " type would justify the opinion—not justifiable in the case of a large supply—that the possibility of their gross faecal contamination is sufficiently remote for their use not to be associated with risk. It is with such waters that the Wilson test for the presence of *Cl. welchii* may turn out to be of decisive value as explained in the text (p. 19) ; the alternative test, " stormy fermentation " of milk, though probably less delicate, may also be used.

We desire to add that we have had the great advantage of the late Sir Alexander Houston's wisdom, knowledge and experience in the preparation of this Memorandum up to the final stage of the work, and to record here our deep sense of the loss caused to us personally and generally to the scientific control of water supplies, by his recent death.

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APPENDIX A.—SAMPLING.

(*a*) *Collection of Sample.*

The following precautions should be strictly observed :—

1. *Sample Bottles* should where possible be obtained from the laboratory performing the examination and should be of good quality glass free from excessive alkali. Preferably the bottle should be of about 230 ml. capacity, and should be provided with a ground glass stopper having an overlapping rim to protect the lip of the bottle from falling particulate matter in the air and from contamination with the fingers during removal of the stopper. The following are the specifications of a good type of bottle :—

Overall height	$6\frac{1}{2}$ in.	=	16.5 cm.
Outside diameter	$2\frac{1}{16}$,,	=	5.25 ,,
Length of neck	$\frac{3}{4}$,,	=	2.0 ,,
Outside diameter of neck	$\frac{7}{8}$,,	=	2.25 ,,
Cubic capacity	230 ml.		

2. *Sterilization*, if performed in the autoclave, should be carried out at a pressure of at least 15 lb. per square inch and maintained for 30 minutes. Before sterilization it is important to insert a strip of paper, about 3 in. by $\frac{1}{2}$ in., between the stopper and the neck of the bottle ; this prevents jamming of the stopper and cracking of the glass on cooling. At least half of the strip should be outside the bottle, so that when the stopper is taken out the paper can be removed without danger of the fingers coming into contact with the rim. A cap of parchment paper should be tied on the stopper and neck and the whole bottle may be wrapped in brown (Kraft) paper if desired. If, after removal of the bottle from the autoclave, the paper wrapping is damp it should be dried off in an oven at a temperature not exceeding 160° C.

Alternatively, sterilization can be effected by dry heat for at least 2 hours at 170° C. This is less likely to cause deterioration of certain kinds of glass.

3. The bottles should not under any conditions be opened until the moment at which they are required for filling with the water and should on no account be previously rinsed out before taking the sample.

4. In collecting the sample, carefully remove the cap covering the stopper and hold the bottle with the hand as far away from the neck as possible. With the other hand very cautiously remove the stopper and hold it in the fingers until the bottle is filled ; carefully replace the stopper and tie down the covering cap. On no account must the stopper be laid down or allowed to touch anything.

The object of this procedure is to prevent any of the sample from coming in contact with the hands or with any surface other than the inside of the sterilised bottle.

5. If the sample is to be taken from a tap, previously ascertain that it is supplying water from a service pipe directly connected with the main. Remove any external fittings, such as an anti-splash nozzle or rubber tube. Carefully cleanse both the outside and inside of the tap, paying particular attention to collections of grease inside the nozzle. Then turn the tap on full and allow the water to run to waste for 2 or 3 minutes in order to flush the interior of the nozzle and to discharge stagnant water in the service pipe. After turning off the tap, cleanse the outer surface with a clean cloth. Next, sterilise the tap either by a blow lamp, or by soaking a piece of cotton wool in methylated spirit, igniting it, and holding it with a pair of tongs close to the nozzle until the whole tap is unbearably hot to the touch. If the tap is out of doors and exposed to air currents, sterilisation is carried out more effectively by means of a blow lamp than by methylated spirit. Cool the

tap by allowing water to run to waste for a few seconds, and fill the sample bottle from a gentle stream of water, taking care to avoid splashing.

Occasionally, when the tap is fully turned on, a slight leak of water may be noticed escaping between the spindle and the gland. This is liable to run down the outside of the tap, and by gaining access to the sample, cause serious contamination. Under such conditions no sample should be taken until the leak is remedied.

Unless it is required to ascertain the bacterial quality of the water contained in a service or house cistern, the sample should always be drawn from a tap connected with the main supply. Cisterns are often inadequately covered, and the water is accessible to dust and to small animals and insects, such as mice, birds, cockroaches, etc., resulting in contamination and bacterial multiplication.

6. In collecting samples direct from a stream, lake, reservoir, spring, or shallow well, the aim must be to obtain a sample that is representative of the water which will be taken for purposes of supply to the consumers. It is therefore undesirable to take samples too near the bank, or too far from the point of draw-off ; if this is by means of a floating arm, the sample should not be taken too deeply. In a stream, areas of relative stagnation should be avoided. Damage to the bank must be guarded against, otherwise fouling of the water may occur.

In taking the sample, the stopper of the bottle should be removed with the fingers of one hand, and the bottle, held by the bottom with the other hand, should be plunged neck downwards below the surface, usually for a distance of about one foot. The bottle should then be rotated till the neck points slightly upwards, the mouth being directed towards the current. If no current exists, as in a reservoir, a current should be artificially created by moving the bottle horizontally in a direction away from the hand. When completely full, the bottle should be brought rapidly above the surface and immediately re-stoppered. Throughout the procedure care should be taken that no water entering the bottle has previously come into contact with the hand.*

7. If the sample is to be taken from a well that is fitted with a hand pump, the pump should be continuously operated for at least 5 minutes before the sample is taken. The mouth of the pump is then heated, preferably by means of a blow lamp, and several gallons of the water then pumped to waste. The sample should be taken by allowing the water from the pump to flow directly into the bottle.

8. If from a well from which pumping is mechanical, the sample should be collected from a previously sterilised tap on the rising main, or from a near-by tap prior to passage of the water into a reservoir or a cistern.

9. In taking a sample from a well from which the water can be raised only by means of pail or can, the pail should be thoroughly cleansed and then sterilised by means of a blow-lamp or by pouring into it boiling water which is allowed to remain in contact for a few minutes and then completely emptied out. After sterilisation and cooling the pail should not be allowed to touch the ground. The pail should then be carefully lowered into the well without touching the sides, and after filling with water should be withdrawn in the same careful manner. The stopper is then removed from the sterile bottle, the bottle being placed on a clean cloth and filled with water by pouring

* Experiments made under severe conditions show that if a sample is taken in the way recommended there is no likelihood of organisms from the hand contaminating the water which enters the bottle, and show further that there is no reason to believe that any particulate matter on the surface of the water will enter the bottle in more than a very small quantity. The results were the same whether the bottle was plunged either neck downwards or bottom downwards.

from the pail. Great care should be taken not to contaminate the stopper with the fingers, etc.

10. Where there is no pumping machinery or other means of raising the water from a well or reservoir in which the level of the water is several feet below the ground surface, special weighted and sterilised containers and bottles can be supplied on request by most well-equipped laboratories.

11. Samples from Public Supplies should be taken from a suitable tap supplying water direct from the main and not from a street hydrant, as it is extremely difficult, if not impossible, to sterilise the latter.

12. It is frequently very desirable, particularly in the case of wells and surface water supplies, that samples should be taken after rainfall.

(b) *Particulars to be Supplied in Submitting Samples.*

No. 1. Name and address of person desiring the examination.

No. 2. Reasons for Examination. If the water is suspected of causing ill-health, the symptoms should be stated.

No. 3. Exact place from which the sample was taken. If from a house tap, state whether drawn through a cistern, or directly from the main.

No. 4. State whether Source is a Well, Spring, Stream or Public Supply.

No. 5. If from a Well state :—

(*a*) Depth.

(*b*) Whether covered or uncovered and the construction of the cover.

(*c*) Whether newly constructed or with any recent alterations which would disturb the conditions of the water.

(*d*) Construction :—

(i) Bricks set dry or in cement.

(ii) Cement or cylinder lined, and whether puddled outside the lining.

(iii) Depth of lining.

(iv) Whether bricked above ground surface. If so, height of coping.

(v) Method of pumping or other means of raising water.

(*e*) Proximity of drains, cesspools, or other possible sources of pollution, and distance from source.

(*f*) Any discoloration of the sides of the well, or other visible indication of pollution.

(*g*) Nature of subsoil and water-bearing stratum.

(*h*) When available, a section or drawing of the well and its general surroundings is desirable.

No. 6. If from a Spring, state :—

(*a*) Stratum from which it issues.

(*b*) Whether sample taken direct from spring or from a collecting chamber. If the latter, mode of construction of chamber.

No. 7. If from a River or Stream, state :—

(*a*) Depth below surface at which sample was taken.

(*b*) Whether Sample was taken from the middle or side.

(*c*) Whether the level of water is above or below the average.

(*d*) Weather conditions at time of sampling, and particulars of any recent rainfall or flood conditions.

(*e*) Observations with reference to any possible sources of pollution in the vicinity and approximate distance from sampling point.

No. 8. Does water become affected in appearance, odour or taste, after heavy rain ?

No. 9. In the case of existing treatment or purification full details should be given.

No. 10. Date and time when sample was taken and despatched.

(c) *Time Interval between Collection and Examination of Sample.*

Owing to the rapid and often extensive bacterial multiplication which may take place in samples of water, the shorter the time elapsing between collection and examination, the more reliable will be the results. All samples should therefore be despatched immediately after collection by the quickest route to the Laboratories, the time occupied in transit being preferably less than 6 hours.

Whenever practicable all samples for bacteriological examination should, as soon as possible, be packed in ice, the ice being placed in a separate closed container and *not* in direct contact with the sample bottle, as this would involve grave risk of contamination of the sample.

APPENDIX B.—APPARATUS.

1. *Sample Bottles.*

To test for alkali, fill the bottle nearly full with distilled water at pH 7.0, to which enough 0.04 per cent. phenol red solution has been added to give a yellow colour. Autoclave for 30 minutes at 15 lb. steam pressure per square inch, with a slip of paper in between the stopper and the bottle, and cool to room temperature. If the glass is satisfactory the colour of the water should still be yellow. A pink or magenta coloration indicates that alkali has been dissolved out of the glass. Since a certain amount of alkali is sometimes dissolved out of quite good glass on the first autoclaving, it is well to repeat the process with all bottles failing to pass the first test. If no alkali is liberated on the second autoclaving, the bottle may be considered satisfactory ; if, on the other hand, the water again becomes alkaline, the bottle should be discarded.

2. *Dilution bottles.*

Preferably the same type of dilution bottle should be used as that described for purposes of sampling. If the bottles are to be used within an hour or two of sterilisation, there is no need to wrap them in paper ; if, however, they are not to be used for a day or more, they should either be wrapped in paper or alternatively the stopper and neck should be covered with metal foil before sterilisation.

3. *Pipettes.*

90 ml. and 50 ml. pipettes should be of the bulb pattern, and should be calibrated to deliver 90 ml. or 50 ml. of water when the water is allowed to

flow down under the influence of gravity whilst the pipette is held vertically with its tip touching the interior of the neck of the dilution bottle.

Both 10 ml. and 1 ml. pipettes should be of the straight-sided pattern, and should be calibrated to deliver the correct amount of water under the same conditions as those of use. The following are specifications for good 10 ml. and 1 ml. pipettes with tapering tips :—

10 ml. pipette.—	Overall length ...	14 ins. = 35 cm.
	External diameter...	10 mm.
	Internal diameter ...	8 mm.
	Graduation ...	1 mark only at 10 ml. level.
	Distance of graduation from tip ...	8 ins. = 20 cm.
	Distance of graduation from top ...	6 ins. = 15 cm.
1 ml. pipette.—	Overall length ...	12 ins. = 30 cm.
	External diameter...	8 mm.
	Internal diameter ...	2½ to 3 mm.
	Graduation ...	1 mark only at 1 ml. level.
	Distance of graduation from tip ...	6–7 ins. = 15–18 cm.
	Distance of graduation from top ...	5–6 ins. = 12–15 cm.

The most satisfactory method of using a 10 ml. pipette is to allow the water to flow down under the influence of gravity, the pipette being held vertically, and then before withdrawal to touch the tip of the pipette against the side of the vessel. With a 1 ml. pipette it is better to blow out the contents, with the tip touching the side of the vessel, allow 3 seconds for drainage, and then blow out the accumulated drop. It is important that all pipettes should be calibrated as delivery, not as container, pipettes. To test their accuracy, distilled water at 18° C. should be sucked up to the mark, and delivered in the appropriate way into a weighing bottle. No pipette should have an error of more than ± 2 per cent. *Before sterilisation every pipette should be fitted with a plug of cotton-wool at the upper end to prevent contamination of the interior by organisms from the mouth.*

4. *Test Tubes.*

These should preferably be of good quality hard glass. Their freedom from soluble alkali should be tested for in the same way as sample bottles. The most satisfactory size for general use in water analysis is 6 ins. × ¾ in., having a total cubic capacity of about 30 ml. In order to economise medium, however, tubes of 6 ins. × ⅝ in., with a cubic capacity of 22 ml., containing an inverted Durham tube 1⅜ ins. × 5/16 in. will be found suitable for sugar medium. All tubes should be plugged with cotton-wool.*

5. *Petri dishes.*

Since a fixed quantity of medium is recommended for use in the plate count, it is important that Petri dishes should be of standard size so as to

* Where media must of necessity be stored for some weeks, 1 oz. screw cap vials with aluminium caps may with advantage be used instead of plug test tubes. (J. E. McCartney, *Lancet*, 1933, Vol. II, p. 433). Before being used for media, the bottles, filled with distilled water, should be autoclaved two or three times to remove soda.

ensure that the surface area and depth of the medium are always constant. The specifications demanded should be :—

Petri Dish.

Top Dish.			
Internal diameter	98 mm.	Tolerance	±1.0 mm.
Height	14 mm.	,,	±1.0 mm.
Thickness of glass	1.8 mm.	,,	±0.2 mm.
Bottom Dish.			
External diameter	94 mm.	Tolerance	±1.0 mm.
Height	17 mm.	,,	±1.0mm.
Thickness of glass	1.8 mm.	,,	±0.2 mm.

The bottom of the dish should be flat ; dishes with a convexity upwards should not be used for counting purposes. Specially large dishes (diameter about 15 cm.) are required for certain processes such as the Wilson sulphite-reduction test.

6. *Cleaning of glassware.*

After use pipettes should be placed in a tall jar containing 2 per cent. caustic soda solution, in which they should be left for at least 2 hours, and preferably overnight. The soda should be removed with a stream of hot water. After being dried on the outside with a glass-cloth, they should be washed through with distilled water ; this may conveniently be done by means of a teat attached to the upper end. They should finally be dried in a hot air oven. Every few weeks, or oftener if necessary, it is well to stand them in a bichromate and acid mixture for 24 hours, followed by very thorough washing in hot water. Test tubes and Petri dishes should be autoclaved. at 15 lb. for 20 minutes, and transferred to hot water. Test tubes should be cleaned with a brush, Petri dishes with a pledget of cotton-wool. The use of a cleaning powder is often of value, but care should be taken to choose a powder that does not scratch the glass.

Test tubes may be boiled in water containing a little washing soda and then left in a dilute acid solution, afterwards being thoroughly washed and drained and allowed to dry gradually, inverted in a draining basket. Petri dishes should be dried and polished with a glass-cloth.

7. *Sterilisation of glassware.*

Glassware should be sterilised by hot air in an oven at 160°–170° C. for 2–3 hours. A temperature of over 170° C. tends to char organic matter and render cotton wool plugs friable and dusty, and should therefore be avoided.

Sample and dilution bottles may alternatively be sterilised by autoclaving. (*See* Appendix A.)

APPENDIX C.—MEDIA.

I. *Nutrient agar.*

Yeastrel*	3 gm.
Peptone†	5 gm.
Washed, shredded or powdered agar	15 gm.
Distilled water	1,000 ml.

* Manufactured by the Brewers' Food Supply Co., Ltd., Edinburgh.

† For this medium and for other purposes for which peptone is used, unless a particular brand is specified, the following varieties appear satisfactory : Allen & Hanbury's, Bacto, British Drug Houses', Fairchild's, Hopkin & Williams', Witte's.

Dissolve the yeastrel and peptone in distilled water in the steamer. Adjust the reaction at room temperature to pH 7.4, using phenol red as the indicator. Weigh out the agar ; if shredded, chop it up, place it in a muslin bag, wash it in running water for 15 minutes, and after squeezing out excess water, add it to the yeastrel peptone mixture. Autoclave at 15 lb. for 20 minutes, and filter through paper pulp* in a Buchner funnel. Egg must not be used for clearing. The pulp is prepared by mashing up small pieces of Postlip filter paper in water by means of a pestle and mortar. A single layer of Chardin filter paper should be laid on top of the Buchner funnel to prevent the pulp being sucked through, and the pulp itself should then be packed down evenly on top of it. The funnel should be inserted into an Erlenmeyer filtration flask fitted with a side piece. A filter pump should be applied to suck through the excess water, which should be poured off through the side piece. The filter, when ready for use, should have a total depth of about 1.5 mm.

The agar should be taken directly from the autoclave and filtered hot, the whole apparatus being kept warm by a surrounding atmosphere of steam. The reaction of the filtrate is tested at 50° C. and adjusted, if necessary, to pH 7.0. Tube in 10 ml. quantities and autoclave at 15 lb. for 20 minutes. The final reaction of the medium at *room temperature* should be pH 7.2. For use, the tubes should either be taken directly from the autoclave or, if they have set, they should be boiled and then cooled down to 50° C. Unless they are to be used within one week of preparation, they should be placed in the cold store to prevent evaporation.

II. *MacConkey Broth* (Single strength).

Commercial sodium taurocholate	5 gm.
Lactose	10 gm.
Peptone	20 gm.
Sodium chloride	5 gm.
Distilled water	1,000 ml.

Steam for two hours and transfer to the ice-chest overnight. Filter in the morning through Chardin paper while still cold. Adjust the reaction to pH 7.4, using phenol red as the indicator. Add about 10 ml. of 1 per cent. aqueous solution of neutral red, distribute in 5 ml. quantities into 6 in. × $\frac{5}{8}$ in. test tubes provided with Durham fermentation tubes, and sterilize in the autoclave at 10 lb. for 15 minutes, or in the steamer for 30 minutes on three successive days. The finished medium should be clear and should have a claret red colour, free from yellow or magenta.

III. *MacConkey Broth* (Double strength) is prepared in the same way as single strength MacConkey broth, using double the above quantities (except water). 10 ml. of this double strength medium is put in each tube (6 × $\frac{3}{4}$ in). If 50 ml. quantities of water are to be tested in MacConkey broth, 50 ml. of this double strength medium should be put into tubes or bottles of greater capacity than 100 ml. Six ounce medicine bottles will be found convenient for this purpose. Each tube or bottle should be provided with a Durham fermentation tube of suitable size.

IV. *MacConkey Agar.*

Commercial sodium taurocholate	5 gm.
Peptone	20 gm.
Sodium chloride	5 gm.
Washed shredded agar	20 gm.
Distilled water	1,000 ml.

Steam until the solids are dissolved. Cool to 50° C., and adjust the reaction

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* A pulp layer of suitable and approximately the same depth for any size of funnel may be obtained by pulping an area of dry filter paper equal to four times the square of the diameter of the funnel.

at 50° C. to pH 7.6 to 7.8. Add egg white, using the albumen of one egg for every 3 litres of medium. Autoclave at 10 lb. for 15 minutes, and filter hot through Chardin paper. Adjust the reaction of the filtrate at 50° C. to pH 7.3, or at room temperature to pH 7.5. Add 10 grams of lactose and about 10 ml. of 1 per cent. neutral red solution (the exact amount required seems to vary with different batches of agar). Mix thoroughly, distribute into flasks, or into milk bottles which can be hermetically sealed, and sterilize in the autoclave at not more than 10 lb. for 15 minutes. For use, melt in the steamer, and pour into Petri dishes, using 15 ml. of medium for each dish.

V. *Wilson-Blair Medium for Cl. welchii.*

Ordinary nutrient agar (3 per cent. agar)	...	100 ml.
20 per cent. solution of anhydrous sodium sulphite	...	10 ml.
20 per cent. solution of commercial glucose	...	5 ml.
8 per cent. solution of ferrous sulphate crystals	...	1 ml.

The Nutrient Agar is melted and the other ingredients added. While still hot, 40 ml. of the medium are added to an equal quantity of the water under examination and poured into a large Petri dish.

VI. *Peptone water* (for indole reaction).

Peptone*	...	10 gm.
Sodium chloride	...	5 gm.
Distilled water	...	1,000 ml.

Steam until the solids are dissolved. Filter hot through Chardin paper, and adjust the reaction at room temperature to pH 7.5. Tube in 5 ml. quantities and autoclave at 15 lb. for 20 minutes. The reaction of the finished medium should be pH 7.4 to 7.6.

VII. *Peptone water sugars.*

To peptone water at pH 6.8 to 7.0, add 1 per cent. of Andrade indicator and autoclave at 15 lb. for 20 minutes. Make a 10 per cent. solution of the required sugar in distilled water, and sterilize in the steamer for 30 minutes. With aseptic precautions add 5 ml. of the sterile sugar solution to 100 ml. of sterile peptone water ; distribute in 4 ml. quantities into sterile 6 in. × $\frac{5}{8}$ in. test tubes containing sterile Durham fermentation tubes, and steam for 30 minutes. The finished medium should have a pH. of 7.4 to 7.6. Incubate for 24 hours at 37° C., and examine for sterility.

VIII. The *Andrade indicator* is prepared by dissolving 0.5 gm. of acid fuchsin in 100 ml. of distilled water. Add 17 ml. of N/1 NaOH solution and leave at room temperature overnight. The following morning the colour should be straw yellow. If it is at all brownish, add a little more N/1 NaOH solution and allow to stand again. This solution is highly alkaline, and consequently media to which it is added should be standardised previously to a pH. of about 6.8.

IX. *Glucose phosphate medium* (for Methyl-red and Voges-Proskauer tests).

Peptone	...	5 gm.
Dipotassium hydrogen phosphate, K_2HPO_4		5 gm.
Distilled water	...	1,000 ml.

Steam until the solids are dissolved ; filter hot through Chardin paper, and adjust the reaction at room temperature to pH 7.5. Add 5 gms. of glucose, mix thoroughly, distribute in 4 ml. quantities into 6 ins. by $\frac{5}{8}$ in. test tubes,

*Experiment shows that Allen & Hanbury's, Bacto, Difco., B.D.H., Fairchild's and Witte's Peptones, are satisfactory for this test, but since experiment also shows that different batches of the same make of Peptone are subject to variation, it is desirable to control the Peptone used with known Indole-producing strains of bacteria.

and autoclave at 10 lbs. for 10 minutes. Test for sterility by incubation at 37° C. for 24 hours.

Note.—In the autoclave the tubes should be placed in a container with a solid bottom to protect them from contact with the boiling water; if they are placed in a wire crate, the medium turns a straw yellow colour.

X. *Litmus Milk.*

Fresh raw milk of low bacterial content—preferably Certified milk—should be allowed to stand in the ice-chest for 18 hours. The cream is removed, and 10 per cent. litmus solution is added to the milk to give a bluish purple colour. Tube in 5 ml. quantities, and steam for 30 minutes on three successive days. Test for sterility by incubation at 37° C. for 48 hours

XI. *Nutrient Gelatin.*

Yeastrel	3 gm.
Peptone	5 gm.
Gelatin (Coignet Gold Label)	120 gm. (in hot weather 150 gm.)
Distilled water	1,000 ml.

Dissolve in steamer, cool to 30° C., and adjust the reaction to pH 7.6 to 7.8 using phenol red as the indicator. Add one-third of the white of an egg to every litre of medium, mix thoroughly, transfer to the steamer, and when thoroughly hot filter through paper pulp in a Buchner funnel, as in the filtration of agar, or through Chardin paper. Adjust the reaction of the filtrate at 30° C. to pH 7.2, tube in 10 ml. quantities, and sterilize in the autoclave at 10 lbs. for 15 minutes. The finished medium should be perfectly clear, and have a reaction of pH 7.2 at room temperature.

XII. *Citrate medium* (for differentiation of coli from aerogenes).

Sodium chloride	5.0 gm.
Magnesium sulphate	0.2 gm.
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 gm.
Dipotassium hydrogen phosphate (K_2HPO_4) ...	1.0 gm.
Distilled water	1,000 ml.

This mixture forms a clear colourless solution with a pH of 6.8. Add 2 grams of citric acid, and bring back the reaction to pH. 6.8 with N/1 NaOH solution. Tube in 5 ml. quantities, and autoclave at 15 lbs. for 10 minutes. Test for sterility by incubation at 37° C. for 24 hours.

XIII. *Endo Medium.**

Meat extract or 0.5 per cent. Laboratory Lemco	1,000 ml.
Peptone (sugar free)	10 gm.
Sodium chloride	5 gm.
Agar	20 gm.

Mix the ingredients and heat in autoclave at 115° C. for 30 minutes; render alkaline by the addition of 10 ml. of 10 per cent. solution of sodium carbonate. Cool to 50° C. and add the white of an egg. Steam for 1½ to 2 hours. Filter while hot through a Chardin paper. To each 100 ml. of filtrate add 1 gm. lactose, 0.05 ml. filtered saturated alcoholic solution of basic fuchsin, and 2.5 ml. freshly prepared 10 per cent. sodium sulphite solution. Steam for 20 minutes on 3 days. When medium is hot it is pink, when cold almost colourless. Should the medium be pink when cold, the amount of sodium sulphite present is insufficient. Medium must be stored in dark.

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* Endo, *Cent. f. Bakt.* 1904, xxxv, 109.

XIV. *Eosin Methylene-Blue Agar** (Levine's modification).

Difco peptone	10 gm.
Dipotassium hydrogen phosphate (K_2HPO_4)	2 gm.
Agar	15 gm.
Lactose	10 gm.
Eosin yellow 2 per cent. aqueous solution ...	20 ml.
Methylene blue 0.5 per cent. solution ...	20 ml.
Distilled water	1000 ml.

Dissolve the peptone, dipotassium hydrogen phosphate, and agar in water by boiling. Make up the loss due to evaporation. Sterilize at 15 lb. for 15 minutes.

Before use, to each 100 ml. of sterile agar add 1.0 gm. of sterile lactose, 2 ml. of 2 per cent. aqueous solution of eosin yellow, and 2 ml. of 0.5 per cent. solution of aqueous methylene blue. No adjustment of reaction nor filtration is necessary. The dyes should be weighed on an analytical balance and added in the order indicated.

XV. *Nitrate Agar.*

Meat extract agar plus 0.1 per cent. potassium nitrate. Adjust reaction to pH 7.5. Sterilize in autoclave for 25 minutes at 115° C.

XVI. *Modified Eijkman Medium.*†

Peptone (Witte)	5 gm.
Beef extract	3 gm.
Dextrose	5 gm.
Water...	1000 ml.

Dissolve the peptone and beef extract in the water. Filter, adjust filtrate to pH 7.2–7.4. Autoclave for 20 minutes at 120° C. Dissolve dextrose in a few ml. of distilled water; steam for 30 minutes. Add sterile solution of dextrose, and tube in sterile tubes containing Durham fermentation tubes. Free steam in the autoclave for 30 minutes.

XVII. *Ehrlich's Reagent.*

Paradimethylamidobenzaldehyde ...	4 gm.
Alcohol (96 per cent.)	380 ml.
Concentrated hydrochloric acid	80 ml.

XVIII. *Metaphenylenediamine Solution.*

Metaphenylenediamine	5 gm.
Water...	100 ml.
Dilute sulphuric acid	one or two drops.

XIX. *Ringer's Solution.* (Full strength.)

Sodium chloride	9.0 gm.
Potassium ,, ...	0.42 gm.
Calcium ,, ...	0.48 gm.
Sodium bi-carbonate	0.2 gm.
Distilled water	1000 ml.

The one-quarter strength solution should be sterilised by autoclaving at 15 lb. for 20 minutes.

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* *J. Infect. Dis.*, 1918, **23**, 43.

† W. L. Williams, R. H. Weaver, and M. Scherago. *American J. of Hyg.*, 1933. **17**, 432.

APPENDIX D.

These tables, computed by McCrady* using a modification of Greenwood and Yule's formula, indicate the probable number of bacteria of the coli-aerogenes group present in 100 ml. of water, as shown by the various combinations of positive and negative results in the quantities used for test.

TABLE I.

Quantity of water put up in each tube.	50 ml.	10 ml.	1 ml.	Probable number of Coli-Aerogenes organisms in 100 ml. of the original water.
No. of tubes used	1	5	5	
Number of tubes giving positive reaction.	0	0	0	0
	0	0	1	1
	0	0	2	2
	0	1	0	1
	0	1	1	2
	0	1	2	3
	0	2	0	2
	0	2	1	3
	0	2	2	4
	0	3	0	3
	0	3	1	5
	0	4	0	5
	1	0	0	1
	1	0	1	3
	1	0	2	4
	1	0	3	6
	1	1	0	3
	1	1	1	5
	1	1	2	7
	1	1	3	9
	1	2	0	5
	1	2	1	7
	1	2	2	10
	1	2	3	12
	1	3	0	8
	1	3	1	11
	1	3	2	14
	1	3	3	18
	1	3	4	20
	1	4	0	13
	1	4	1	17
	1	4	2	20
	1	4	3	30
	1	4	4	35
	1	4	5	40
	1	5	0	25
	1	5	1	35
	1	5	2	50
	1	5	3	90
	1	5	4	160
	1	5	5	180+

NOTE.—The above most probable numbers, from 0 to 20 are correct to the nearest unit; above 20 are correct to the nearest 5.

* *Canadian Pub. Health J.*, 1918, 9, 201.

TABLE II.

Quantity of water put up in each tube.	10 ml.	1 ml.	0.1 ml.	
No. of tubes used	5	5	5	
Number of tubes giving positive reaction.	0	0	0	0
	0	0	1	2
	0	0	2	4
	0	1	0	2
	0	1	1	4
	0	1	2	6
	0	2	0	4
	0	2	1	6
	0	3	0	6
	1	0	0	2
	1	0	1	4
	1	0	2	6
	1	0	3	8
	1	1	0	4
	1	1	1	6
	1	1	2	8
	1	2	0	6
	1	2	1	8
	1	2	2	10
	1	3	0	8
	1	3	1	10
	1	4	0	11
	2	0	0	5
	2	0	1	7
	2	0	2	9
	2	0	3	12
	2	1	0	7
	2	1	1	9
	2	1	2	12
	2	2	0	9
	2	2	1	12
	2	2	2	14
	2	3	0	12
	2	3	1	14
	2	4	0	14
	3	0	0	8
	3	0	1	11
	3	0	2	14
	3	1	0	11
	3	1	1	14

Probable number of Coli-Aerogenes organisms in 100 ml. of the original water.

TABLE II.—*continued.*

Quantity of water put up in each tube.	10 ml.	1 ml.	0.1 ml.	
No. of tubes used	5	5	5	
Number of tubes giving positive reaction.	3	1	2	17
	3	1	3	20
	3	2	0	14
	3	2	1	17
	3	2	2	20
	3	3	0	17
	3	3	1	20
	3	4	0	20
	3	4	1	25
	3	5	0	25
	4	0	0	13
	4	0	1	17
	4	0	2	20
	4	0	3	25
	4	1	0	17
	4	1	1	20
	4	1	2	25
	4	2	0	20
	4	2	1	25
	4	2	2	30
	4	3	0	25
	4	3	1	30
	4	3	2	40
	4	4	0	35
	4	4	1	40
	4	5	0	40
	4	5	1	50
	5	0	0	25
	5	0	1	30
	5	0	2	40
	5	0	3	60
	5	0	4	75
	5	1	0	35
	5	1	1	45
	5	1	2	60
	5	1	3	85
	5	2	0	50
	5	2	1	70
	5	2	2	95
	5	2	3	120

Probable number of Coli-Aerogenes organisms in 100 ml. of the original water.

TABLE II.—*continued.*

Quantity of water put up in each tube.	10 ml.	1 ml.	0.1 ml.	
No. of tubes used.	5	5	5	
Number of tubes giving positive reaction.	5	2	4	150
	5	2	5	175
	5	3	0	80
	5	3	1	110
	5	3	2	140
	5	3	3	175
	5	3	4	200
	5	3	5	250
	5	4	0	130
	5	4	1	170
	5	4	2	250
	5	4	3	300
	5	4	4	350
	5	4	5	450
	5	5	0	250
	5	5	1	350
	5	5	2	600
	5	5	3	900
	5	5	4	1,600
	5	5	5	1,800+

Probable number of Coli-Aerogenes organisms in 100 ml. of the original water.

NOTE.—The above most probable numbers from 0 to 20 are correct to the nearest unit. From 20 to 200 are correct to the nearest 5. Above 200 are correct to the nearest 50.

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