THE TECHNIQUE OF STERILITY TESTING

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The expanding use in medicine of drugs administered by parenteral routes has brought with it an increase in application of aseptic techniques and consequently in the development of reliable tests for sterility. Sterility tests were introduced in the British Pharmacopœia for the first time in 1932. Before this date the Regulations[1] made under the Therapeutic Substances Act, 1925, had specified such tests for certain vaccines, toxins, sera and similar products as well as for insulin and arsphenamines. From time to time other substances such as surgical ligatures, blood products and the antibiotics were added, and all were consolidated into the new Regulations[2] of 1952. The purpose of the prescribed tests is to detect the presence of living aerobic and anaerobic micro-organisms; specifications, but not formulæ, for suitable culture media are outlined in the Regulations and in the B.P.

The mode of all tests for sterility on preparations for injection is based on four principal premises; 1, that proper aseptic techniques have been carried out during processing, 2, that the procedure followed in making the test eliminates as far as possible the risk of introducing accidental contamination at this stage, 3, that the culture medium used is capable of detecting "small numbers of the commoner contaminating micro-organisms"[2], particularly pathogenic types or those which might cause spoilage of the medicament, and 4, that any bacteriostatic substance in the preparation is sufficiently diluted or neutralised to render it inactive.

On the first premise, it is clearly a prerequisite that before a preparation is submitted to a test for sterility it must have been subjected to such a treatment as can be reliably expected to yield a sterile product. The exclusive purpose of the test is to check that the approved process has been carried out satisfactorily; the test cannot of itself check that the process is satisfactory. On the second premise, it is recognised that an occasional growth could occur in the test which either originated from the injection or was introduced adventitiously during testing. To check this, the Regulations allow a second test, and, if necessary, a third test to be made, provided the organisms found in the first and second tests are manifestly different. The material is satisfactory only if one of the tests is completely free from growth. It follows, therefore, that the manipulative technique of testing should be as methodical as possible to minimise contamination from outside sources. A training scheme for operators in aseptic techniques was outlined by Coulthard[3], who also emphasised the importance of continuous bacteriological control of the premises, apparatus and methods employed for successful work of this nature.

Technique of Sterility Testing

Methods employed. In spite of the importance of sterility testing, comparatively little has been published on the actual techniques of carrying out the tests. Berry[4] drew attention to this deficiency and

Hopkins[5] has attempted to outline some of the precautions necessary. Whether one is required to carry out large numbers of tests regularly or only an occasional test, the principle and the precautions to be followed are essentially the same—the conditions under which the tests are made must be so designed and controlled as to eliminate outside contamination.

To this end the work should be carried out in a small "sterile" room —a room 6 ft. square is adequate—supplied with a flow of sterile-filtered air, the operations being conducted under an inoculating screen, also fitted to allow a controlled flow of sterile air. The room should contain only a necessary minimum of equipment so that it can be completely and easily disinfected. The sterile air supply must always be directed to carry contamination away from the site of operation. A regular "drill" of disinfecting the room and screen, and of assembling the test samples, sterile syringes and culture media must be followed, and the operators concerned should be dressed in sterilised gowns, headgear and rubber gloves and should disinfect their hands and arms with a non-irritating disinfectant, such as one of the proprietary chloroxylenol preparations. The tests must be made with a minimum of movement and air disturbance by the operators; each tube or bottle of medium must be "flamed" before opening and after inoculating, and the exteriors of the test ampoules or vials must be rendered free from contamination. This may be effected by immersing in 70 per cent. ethanol containing 1 per cent. of hydrochloric acid, and draining. Syringes are preferred to pipettes for inoculating the test media; they should be changed with each batch of material, and several times between batches, to avoid contamination. When ampoules are to be tested, their tops must be removed carefully with the aid of a small file; with vials the rubber cap should not be removed but samples should be withdrawn through the cap by means of a syringe. To facilitate this withdrawal, it is useful, before inserting the syringe into the cap, to draw into the syringe sufficient sterile air to replace the sample volume.

An improved testing method described by Royce and Sykes[6] dispenses with the majority of the preliminary preparations usually associated with aseptic manipulations and virtually eliminates the chances of introducing contaminations during testing. It uses a hermetically sealed screen and depends on the sterilising action of gaseous ethylene oxide. Obviously, access of the sterilising gas to the test sample or to the culture media must be excluded, hence the method can be used only when the samples are in containers such as ampoules, vials or bottles sealed from the air, and when the test media are also in screw-capped containers. The use of screw-capped containers in place of cotton wool plugged tubes for culture media was advocated some years ago[7], and to-day they are in common use, as for example in the well-known "McCartney bottles". The screen is fitted with long-sleeved rubber gloves fixed in such a position that the hands and arms are relatively free to carry out the normal operations within the screen. For use, it is loaded with the test samples, the sterile culture media, sterile syringes and all other necessary ancillary equipment. The volume of gaseous ethylene oxide introduced is 12·5 to 15 per cent. (v/v) and the screen is sealed and left for 18 to 24 hours during which

sterilisation of the whole of the exposed surfaces takes place. The ethylene oxide is removed by aspirating sterile-filtered air through the screen after which the inoculations of the test samples into the media are carried out normally. Provided adequate care has been taken in preparing the screen and its contents and in introducing the ethylene oxide, the danger of introducing accidental infection from outside sources is practically non-existent.

An entirely different method for making tests on liquid preparations is the filtration technique of Davies and Fishburn[8] designed primarily to overcome the carry-over of inhibitory concentrations of bacteriostatic substance into the test media. It has a number of commendable points, but the risk of contamination during manipulation cannot be discounted[9]. The procedure consists simply of passing the test sample aseptically through a small sterilised asbestos filter-pad and then culturing the whole of the pad in nutrient broth. The authors recommend using two pads, one for the aerobic test and one for the anaerobic test.

Testing Oils. The problem of freeing bacteria from an oily coating to allow them to proliferate in the surrounding nutrient medium is a difficult one, and for this reason the sensitivity of sterility tests on oils is much lower than on aqueous preparations. A number of media, including milk, gelatin and agar "shake" tubes, have been tried in the past with variable success. Davies and Fishburn[8] claimed that their filtration method is particularly suitable for this purpose and much more sensitive because the pads can be washed free from the oil by quickly rinsing through with light petroleum, thus removing the protective oily coating from any organisms present. Bullock and Booth[10] did not find any increased sensitivity by the filtration method and considered one of the most satisfactory procedures to be that of shaking the broth-oil mixture on several occasions during the incubation period. This is advocated in the B.P. 1953, and in the U.S.P. XIV[11].

Control Tests. Whatever testing system is employed, it is essential that the technique is continuously and adequately controlled. Timoney[12] mentioned the need for control tests on the media used, and Foord, Crane and Clark[13], discussing the testing of food containers, considered the hazards of aerial contamination to be so great that when examining for "absolute sterility" they felt it necessary to have at least one control for every three containers tested. Control infers proving that (*a*) the medium is sterile, (*b*) the syringes employed for inoculating the test samples are sterile, and (*c*) the testing procedure is carried out aseptically. The first of these is checked by incubating samples from each batch of medium at 37° C. and at about 25° C. for several days; the second can conveniently be checked by drawing broth into each syringe immediately before use, and injecting half into aerobic broth and half into anaerobic broth and incubating; the third is checked by making at intervals actual sterility tests under normal conditions, but with materials which have been sterilised in the laboratory by a reliable method. Water or saline sterilised in the autoclave in ampoules or vials is a convenient check material for liquid preparations, and sodium chloride sterilised in the hot-air oven is suitable for solid preparations.

Check tests of the foregoing type give valuable information on the reliability of the operational methods employed; as such they are indispensable features of a complete sterility testing system.

Temperature and Time of Incubation. After the sterility test media have been inoculated with the appropriate samples, they are incubated at 37° C. for five days. This temperature is chosen because it is the optimum for most types of micro-organism likely to be encountered, but there are many organisms capable of causing trouble which do not grow at this temperature. A "Memorandum on Avoidable Meningitis" issued with the authority of the Ministry of Health and reported in *The Pharmaceutical Journal*[14] discussed the causes of infection after spinal injections and found that "the organisms most frequently incriminated are *Ps. pyocyanea* and related organisms which can multiply in water at room temperature. . . . Some of the water bacteria fail to grow in ordinary media incubated at 37° C.". Wetterlow, Kay and Edsall[15] also commented on missed contaminations of psychrophilic organisms, that is, organisms incapable of growth at 35–37° C., due to incubation of tests at this temperature range. Such organisms are said to be found fairly frequently as contaminants in animal sera, blood, and other products of biological origin and in preparations containing no preservative; it is on record[16] that coliforms, which escaped detection at 37° C. but grew well at 23° C. have been recovered from human albumin. The obvious answer is to institute tests incubated at 20–25° C. as well as at 37° C. The U.S.P. follows this principle, albeit in a somewhat accidental and indirect manner, by including tests for moulds to be incubated at 25° C. A Sabouraud liquid medium is specified for this purpose, but it would certainly allow the growth of many psychrophils.

An incubation period of five days is required to allow organisms, present in only small numbers and possibly "damaged" by adverse bacteriostatic conditions, to grow and produce a visible turbidity. The Therapeutic Substances Regulations of 1927[1] specified seven days. Several investigators have commented on the long dormancy periods required for proliferation by some bacteria after a mild disinfectant or heat treatment; in particular, Burke, Sprague and Barnes[17] working with aerobic organisms including *Escherichia coli*, and Esty and Meyer[18] working with anaerobes observed delays in germination of periods of from several days up to a year or more. A useful review on this aspect of the subject was presented in 1930 by Morrison and Rettger[19], and later Wynne and Foster[20] described studies on the physiology of spore germination in *Clostridium botulinum* cultures.

It is desirable during the incubation period to examine the tests on several occasions, as it is not unknown for an organism to grow into a visible turbidity, and then settle leaving a clear supernatant broth which unless examined carefully, could give a false impression of sterility.

Culture Media Used in Sterility Testing

Criteria for Media. The purpose of a test for sterility is to ensure that the medicament is free from all contaminating micro-organisms. However desirable this may be in theory it cannot be achieved in practice

because of the wide, and sometimes specific, nutritional and temperature requirements of different bacterial and fungal types. Therefore, a compromise has to be made, and, to quote the Therapeutic Substances Regulations[2], "The tests . . . shall be made in a fluid medium, which, as sterilised for use, shall be capable of promoting the vigorous growth of the commoner contaminating micro-organisms, both aerobic and anaerobic". Miles[21] suggested that *Staphylococcus aureus* spp. should be included in the range of micro-organisms used for testing aerobic media, and *Clostridium œdematiens* for anaerobic media.

Not only is it a criterion that a satisfactory medium must support the growth of as wide a range of bacterial types as possible, but also that it shall support their growth when inoculated in small numbers. Such tests should be comparative with an approved medium and it is important to remember that the choice of the volume of medium in the container may be significant. Thus, growth from the same small inoculum may take place more readily in 10 ml. than in, say, 500 ml. of the same broth.

The most generally recommended media are those made with an extract or enzymic digest of meat. Such media, containing peptones and a range of amino-acids as well as other "growth factors" are sufficiently nutrient for most micro-organisms. However, the more fastidious streptococci and pneumococci might not grow readily in them, and certainly tubercle bacilli would not be detected within the limited five-day incubation period; neither could the viruses proliferate. But, it is reasonable to assume that such organisms could not be introduced as contaminants in pure culture. They would almost certainly be in association with the commoner staphylococci or Gram-negative organisms, all of which should grow readily in the normal media.

Choice of Media. The B.P. 1953, states that the medium used for aerobic organisms "either consists of meat extract containing a suitable concentration of peptone or is prepared by the enzymic digestion of protein material", and that for anaerobic organisms is similar but with the addition of either heat-coagulated muscle or about 0·05 per cent. of agar with a substance to reduce the oxidation-reduction potential of the medium. One of the first digest media, as distinct from simple meat infusions, was that described by Douglas[22], made by a short period tryptic digestion at 45° C. of bullock heart. Later, Hartley[23] modified the process by using an overnight digestion at 37° C. of lean horse flesh. Subsequently, several further modifications have been suggested, mainly in relation to the source and quantity of meat. A digest of bullock heart using half the quantity of meat of the Hartley formula has been found sufficiently nutritive to allow the growth of the pyogenic streptococci[24]. Barnes[25] has shown recently that the method of extracting the enzyme from pancreas determines to a large extent the nutritive properties of a tryptic digest broth. Alcoholic extracts yielded more satisfactory broths than did acid-extracted or commercial trypsins. She considered the difference to be due not to growth factors provided by the alcoholic extract but to supplementary non-proteolytic enzymes, e.g., amylase and lipase, which would not be present in the acid extract. Papain can be used in the

place of trypsin[26]. In this case the digestion is usually carried out at a higher temperature for a shorter period—60° C. for 4 to 6 hours is commonly employed. It gives a more complete digestion so that a smaller amount of meat can be used to produce the same volume of broth. In Table I, the nutrient properties of typical trypsin and papain digest broths are compared in terms of the rate of growth of small inocula of *Staph. aureus* in 10 ml. of medium at 37° C. Both types of media give a relatively short lag-phase of some two hours, after which the organisms grow with normal rapidity. By way of contrast, an unsatisfactory trypsin medium is included.

Because of possible variations in the source and quality of the meat used and also in the rate and type of digestion, it is convenient to standardise digest media by their amino-acids content and biuret reaction. But this alone does not assess the nutritive properties of the medium. Many other growth factors occurring naturally in meat must also be present.

TABLE I

RATE OF GROWTH OF *Staph. aureus* IN MEAT DIGEST BROTHS

Period of incubation of inoculated broth (hrs.)	Viable bacteria per ml. in :—				
	Trypsin broth A	Trypsin broth B	Papain broth A	Papain broth B	Trypsin broth (unsatisfactory)
Initial inoculum	6	3	6	7	6
1½	6	8	8	23	4
2	7	9	16	49	7
2½	22	11	40	136	1
3	61	51	86	310	1
3½	183	106	225	c 1000	2
4	500	223	500	>1000	1
4½	c 1000	500	c 1000	>1000	0

All tubes were incubated at 37° C.

For this reason the quantity of meat used in a digestion must be standardised and must not be reduced too far, otherwise the resultant broth will be deficient in these growth factors. This is well illustrated in some experiments made on a papain digest of the residues of meat after trypsin digestion. Both the trypsin and the papain broth gave satisfactory amino-acid titration values, but the papain digest, being made from exhausted meat tissues, was deficient in soluble growth factors and consequently did not adequately support the growth of staphylococci.

Arising from anticipated war-time shortages of meat, Gladstone and Fildes[27] devised a medium without peptone or meat extract. It is made with acid-hydrolysed and tryptic-digest caseins, yeast extract, sodium glycerophosphate, sodium lactate, and a small amount of glutamine. It is claimed to support the growth of organisms met with in medical bacteriology even better than do meat media, and to be cheap to make. Brewer[28] also suggested a meatless medium made by the papain digestion of vegetable meals such as those of cotton seed, peanut, soya-bean, whole and sprouted grains and other seeds. He found it equal to, or more satisfactory than, meat media for general culture purposes, but it does not seem to have been adopted for normal use.

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In preparing media for routine use, it is important to check each batch for its ability to support growth, because sometimes inhibitory contaminating substances may inadvertently gain access during processing. Attention was first drawn to this by Dubos[29], who observed the phenomenon in certain commercial peptones. He was not aware of the nature of the substance, but found it to cause bacteriostasis only under oxidising conditions and not at all under reducing conditions. Later O'Meara and Macsween[30] investigated the reasons for the failure of staphylococci to grow in certain meat digest and peptone media. They attributed it to copper dissolved from the metal pans during the digestion process. Marked inhibition was exhibited by as little as 4 p.p.m. of copper, a concentration "of the order commonly found in routine media", but the effect was not direct. It could only be demonstrated by heating the medium and then keeping it for some time; and it was abolished by reheating. Examining the action further, Woiwod[31] attributed it to the formation in the medium of colloidal copper sulphide during the heating process. The mechanism of the action is enigmatical, since no inhibition occurs in media heated in sealed containers, and it is confined to staphylococci and other Gram-positive organisms. Colloidal sulphur and the sulphides of iron and manganese are also inhibitory but to a much less extent. In this connection, a recent report on the chemical analyses of different peptones[32] is of interest.

In 1929, Dubos[33] observed losses in the nutritive power of media after keeping them for several weeks. On the basis that all micro-organisms grow better under somewhat reduced oxidation-reduction potential systems, he was able to show that it could be restored by reducing the oxidation-reduction potential of the system by heating, by reducing with hydrogen or by adding cysteine or blood. In a later publication[29] he showed that reduced thiol compounds were always effective and so advocated the addition of thioglycollic acid, or its sodium salt.

Other investigators have reiterated the value of thioglycollate broth. Thus, Reid and Bowditch[34] believed it to be superior to ordinary broth or blood media for diagnostic purposes, and Marshall, Gunnison and Luxen[35], Fay and Blubaugh[36], Graydon and Biggs [37] and Berry[4] found it more effective in sterility testing, particularly with biological materials containing a mercury compound as the bacteriostatic agent. Brewer[38] also found it advantageous in a medium containing "pork infusion solids", peptone and a small amount of agar for detecting and cultivating anaerobic organisms. The U.S.P. adopted a modification of the so-called "Brewer's medium" for use in sterility testing, the modified medium containing L-cysteine, yeast extract, casein digest, glucose and sodium thioglycollate, with agar as an optional addition to give a semi-solid fluid. The B.P., 1953, now recommends a similar medium. The obvious advantages of a thioglycollate medium are that it is clear for ease of reading, it detects aerobic and anaerobic organisms simultaneously, and it neutralises the action of certain bacteriostatic agents. Care must be taken, however, to use freshly made medium, as it can become inhibitory due to deterioration on storage[39,40]

The inclusion of agar to increase the viscosity of the medium was first advocated by Hitchens[41] in 1921, who claimed that a 0·1 per cent. concentration gave greater sensitivity in the detection of both aerobic and anaerobic organisms. This was confirmed later by Spray[42] working with anaerobic organisms alone. Falk, Bucca and Simmons[43] investigated the phenomenon more closely using the hay bacillus, staphylococci, streptococci, Gram-negative organisms and diphtheroids, and found the optimum concentration of agar to range between about 0·06 and 0·25 per cent. This was most marked when the inocula of organisms were small, that is of the order of 10 per ml. and a concentration of 0·1 per cent. was finally recommended.

Media for Anaerobic Organisms. For the cultivation of anaerobic organisms any ordinary nutrient medium may be used at a low oxidation-reduction potential. Satisfactory reducing conditions can be attained in practice by excluding aerial oxygen with a soft paraffin or other seal, or by adding to the medium reducing compounds such as alkaline sulphides, pyruvic acid, ascorbic acid, cysteine, glutathione or thioglycollic acid. As far back as 1917, Douglas, Fleming and Colebrook[44] suggested putting rusty nails in ordinary culture media to encourage the "aerobic" growth of *Clostridium butyricum* and *Cl. œdematiens*, and much later Hayward and Miles[40] took this further by suggesting the use of strips of sterilised mild steel dropped into tubes of media as required. The iron did not adversely affect the nutritive properties of the media, which were considered superior to thioglycollate media in that the latter tend to deteriorate on storage. The idea was put forward in the first place in lieu of the McIntosh and Fildes jar as a means of studying anaerobic bacteria, but it might easily be adapted for sterility testing. The principal disadvantage is that the iron acts purely in a reducing capacity and has no power to inactivate bacteriostatic agents, whereas other reducing agents, such as meat and thioglycollate, act in the dual capacity. *En passant*, a specific test used by Pulvertaft[45] for detecting tetanus spores in sanitary towels was to place each towel in a sterile Kilner jar, cover it with boiling broth, immediately screw down the lid and incubate at 37° C. Such a test obviously could only be used for detecting heat-resistant spores.

The classical medium employed for many years in sterility testing for anaerobic organisms is based on Robertson's meat medium, that is, a nutrient broth with a deep layer of minced, lean meat at the bottom; any meat can be used, but heart muscle is preferred to flesh[46]. The meat is prepared by pre-cooking in a weak alkali solution; raw meat can be used but it tends to give an opalescent and "stringy" appearance to the final medium. Such a medium when freshly sterilised is sufficiently oxygen-free to allow the growth of obligate anaerobes, but it fairly quickly re-dissolves oxygen on standing. Marchal[47] observed that, for maximum sensitivity in detecting small numbers of anaerobic bacteria, not only is it desirable to have meat present, but also the medium should be covered with a liquid paraffin seal. It is, therefore, recommended that for sterility testing meat media should be sealed with a layer of soft paraffin and be heated and cooled immediately before use. The function of the

seal is to reduce the rate of dissolution of oxygen and the heating is to drive off any oxygen which might have gained access. The relative capacities of meat media with and without a seal to support the growth of two species of *Clostridium*, *Cl. welchii* and *Cl. sporogenes*, are illustrated in Table II.

TABLE II

GROWTH OF ANAEROBIC ORGANISMS IN MEAT AND THIOGLYCOLLATE MEDIA

Dilution of culture inoculated (1 in −)	Growth* of *Cl. welchii* in:—				Growth* of *Cl. sporogenes* in:—			
	Meat medium without seal	Meat medium with seal	U.S.P. thioglyc. medium	Meat medium +0·1 per cent. thioglyc.	Meat medium without seal	Meat medium with seal	U.S.P. thioglyc. medium	Meat medium +0·1 per cent. thioglyc.
1×10^{7}	+	+++	+++	+++	0	+++	+++	+++
3×10^{7}		++	+++	+++		+++	+++	+++
1×10^{8}	0	+	+++	+	0	0	+++	+++
3×10^{8}		+	+	++		+	+	+++
1×10^{9}	+	0	0	+	0	0	0	+
3×10^{9}		0	0	0		0	0	0
1×10^{10}	0	0	0	0	0	0	+	+

* 50 ml. tubes of medium used, and inoculated with 1 ml. of diluted culture; 3 tubes inoculated at each test level; +++, ++, + and 0 = no. tubes showing growth.

The function of the meat in the medium is somewhat obscure. Lepper and Martin[46] suggested that it carries natural reducing systems in the form of glutathione and thiol groups and of unsaturated fatty acids, which are catalytically oxidised by the hæmatin present in the denatured muscle protein. Knight[48] suggested in addition that the meat fibres provide a nidus to assist in initiating proliferation of the organism. Evidence to support the latter point had earlier been provided by Marchal's observation[47] that asbestos could equally well take the place of meat in the medium.

According to Knight[48], a meat medium has only a small reducing capacity which is gradually and irretrievably lost on storage due to the slow natural absorption of oxygen. It is, therefore, desirable to add suitable agents to increase the reducing capacity. Of the several agents possible, thioglycollic acid seems to be the most suitable. It had been suggested as early as 1926 by Quastel and Stephenson[49] as a means of cultivating anaerobes; Fildes[50] used it in his studies of anaerobic bacteria, and McClung[51] advocated its inclusion in infusion media for the large scale cultivation of anaerobes. As stated earlier, Brewer[38] independently devised a thioglycollate medium for the "aerobic" cultivation of anaerobes, and such media are frequently referred to by his name. The relative growth capacities of thioglycollate and of meat media are illustrated in Table II. Thioglycollate media have been commented upon favourably by many other workers e.g.[4,29,33,38]. They have the advantage that they do not require a seal, they do not need to be re-heated prior to use, and they retain their anaerobic properties for long periods. Moreover, they can be used facultatively because they allow the growth of micro-aerophilic and aerobic organisms, and the thioglycollic acid combines with and inactivates some bacteriostatic agents.

Media for Moulds and Yeasts. As well as tests for organisms growing at 37° C., the U.S.P. requires tests to be made for the presence of moulds and yeasts by incubating inoculated media at 22° to 25° C. for at least 14 days. The British regulations do not require such tests, but they are commonly applied, particularly to injections known to be subject to contamination by mould, yeast or any bacteria with optimum growth temperatures below 37° C.

The medium specified in the U.S.P. XIII was a honey medium made with enzyme-digested casein and 6 per cent. of honey, but in the next revision it was changed to a modified Sabouraud liquid medium containing peptone and 2 per cent. of dextrose, adjusted to pH 5·7. Several other media could be used satisfactorily, such as wort agar or malt agar or plain glucose agar or glucose broth. The primary requirements are that the medium should contain carbohydrate, that the pH value should be on the acid side, that is, ranging between pH 5 and pH 6, and that the incubation temperature should be at about 25° C.

Inactivating Agents

Many of the medicaments administered by injection to-day are themselves antibacterial, and their numbers are increasing as a result of the introduction of the newer antibiotics and chemotherapeutic agents. Other injections, particularly those dispensed in multidose containers, contain an added bacteriostatic agent. Those most generally added to injections are phenol, cresol, chlorocresol, chlorbutol, benzyl alcohol, esters of *p*-hydroxybenzoic acid, quaternary ammonium compounds and compounds of mercury such as phenylmercuric nitrate and thiomersal. Unless due care is taken the antibacterial properties of the preparation can be carried over in the sterility test, resulting in false-negative readings being obtained from samples which are actually contaminated. To meet this, the Therapeutic Substances Regulations[2] and the U.S.P. XIV[11] specify that when a bactericidal or bacteriostatic agent is present either it shall be diluted in the test with such a volume of medium as will render the agent ineffective, or it shall be treated with a suitable inactivating agent.

Inactivation by Dilution. The phenolic substances can all be dealt with by a simple dilution in the sterility test medium. The earlier Regulations[1] required their final concentrations to be less than 0·01 per cent., and, since their concentrations in the injections do not exceed 0·5 per cent., this was readily achieved by inoculating each 1 ml. of the test sample into 50 ml. of medium. It has been suggested[39] that a 0·5 per cent. solution of phenol or chlorbutol even when diluted fifty times is still capable of inhibiting germination of spores, and Flett and others[52] have produced evidence to show that *Staph. aureus* and *Salmonella typhi* can be revived after treatment with dilutions of phenol hitherto considered lethal (e.g., 1 in 65 said to kill in 10 minutes) by adding activated charcoal or ferric chloride to the recovery medium. There seems to be little support, however, for either of these views, in particular, neither Tilley[53] nor Jacobs and Harris[54] were able to confirm the latter.

As well as the antibiotics and sulphonamides, a number of other substances used in injections possess antibacterial properties. Thus, Davies and Fishburn[8] recorded "bacteriostatic concentrations" of hexobarbitone and phenobarbitone at a dilution of 1 in 10^3, thiopentone at 1 in 10^3 and mepacrine methanesulphonate at 1 in 10^4; Booth[5] advised diluting all barbiturates to a final concentration of less than 0·2 per cent. in the test broth and also drew attention to the need for reducing the pH value of some of the broth dilutions of the sodium salts which may be as high as pH 9. Kohli and others[56] and Gupta[57] also drew attention to the bacteriostatic properties of a number of drugs commonly administered by injection, and recommended suitable dilutions to be employed in testing. Adequate dilution will overcome the inhibitive properties of these compounds, but it is an essential part of sterility testing to ensure that the dilution has been sufficient by inoculating control tubes with small numbers of test organisms and observing their growth on incubation.

Inactivation of Mercurial Preservatives. Unlike the phenolic compounds, the compounds of mercury used as bacteriostatics are not inactivated by simple dilution in nutrient broth. They are adsorbed on the bacterial surfaces and can only be satisfactorily neutralised by chemical action. It has been suggested that adding charcoal to the medium is effective[58], but sulphydryl compounds have proved more acceptable. Cysteine has been used[59,60] but again, thioglycollic acid is generally preferred[39]. A broth containing 0·05 per cent. of thioglycollate is said to give more satisfactory results in testing sera containing thiomersal[37], and it is reported to give a greater number of positive responses from vaccines inoculated with staphylococci[36].

The value of thioglycollic acid in sterility test media has already been discussed. In the particular case of the mercurials, it would seem to act in two ways: (*a*) by neutralising the mercurial by chemical combination, and (*b*) by encouraging the growth of bacteria as a result of inducing a more favourable oxidation-reduction potential.

Inactivation of Arsphenamines. The various arsphenamines administered by injection all become highly toxic on exposure of their solutions to the air, such that dilutions up to 1 in 100,000 may be lethal or inhibitory to the commoner bacteria—Berry and Jensen[61] have put the figure even higher in some cases, finding the Gram-positive bacteria more sensitive than the Gram-negative. Ascorbic acid in the proportion of one part to three parts of neoarsphenamine or Mapharsan is reported to delay oxidation for at least forty-eight hours[62]. Minced meat was also found to mitigate the toxicity developed during testing, presumably because of the thiol compounds naturally present, and this was used for many years in the author's laboratory. However, a more sensitive and reliable medium was sought, and finally a meat and thioglycollate medium was chosen, on account of its efficacy in supporting the growth of small bacterial inocula, its stability and availability. The medium employed was a tryptic digest broth containing 0·4 per cent. of sodium thioglycollate and a layer of minced meat[63]. Meat was included because it gave slightly more favourable conditions for *Staph. aureus* and *Bacillus subtilis*. Berry

and Jensen[61] also concluded that thioglycollic acid or dimercaprol could be used satisfactorily. They also made the novel suggestion that where it is difficult to tell whether there is growth or not the differentiation might be made by observing changes in pH value.

Inactivation of Sulphonamides. The sulphonamides are frequently applied topically to open wounds; hence it is essential that they, as well as other powders thus applied, shall be free from contaminating bacteria, especially pathogenic anaerobes, and that adequate tests be applied to confirm it. There are a number of cases on record of deaths from tetanus following the application of non-sterile powders[64–67].

The antibacterial activities of the sulphonamides are known to be influenced considerably by other substances present in solution. Thus, *p*-aminobenzoic acid, methionine, purines, casein, albumin, meat extract, blood and serum all reduce their activities to a greater or lesser extent. Much has been written on the influence of culture media constituents and on the effect of different sized inocula of bacteria in relation to their resistance and growth in the presence of sulphonamides[67–72], all of which is, in effect, an expansion of Fleming's findings[73] that small inocula are more easily inhibited than larger ones and that adding peptone to media tends to inhibit bacteriostasis. On the basis that minced meat contains natural sulphonamide inhibitors, it has been used successfully by the author in routine sterility testing. Liver infusion media are reported to be of little value in inactivating sulphathiazole, and human serum to be more effective than human plasma, rabbit blood or horse serum[74]. The sulphonamide antagonists naturally occurring in most media are apparently neutralised by horse red blood cells but not by those of other animals[75].

Since the now classical work of Woods[76] in discovering the antagonising effect of *p*-aminobenzoic acid on sulphonamides, the most reasonable procedure seems to be to add a measured amount of the acid to culture media as required. It has been claimed[77] that Brewer's medium contains sufficient sulphonamide inhibitor to inactivate 50 mg. of sulphanilamide per 20 ml. of medium, but Brewer himself, as quoted by Long[78], preferred to add *p*-aminobenzoic acid. The amount required is small, since one molecule of the acid is said to be sufficient to antagonise 5000 molecules of sulphanilamide[76]. In practice, 250 ml. of a 0·01 per cent. solution of *p*-aminobenzoic acid in broth is sufficient to neutralise 5 g. of sulphanilamide or sulphathiazole.

Inactivation of Penicillin. Although solutions of the penicillin salts are labile, their stabilities over a few days are sufficient to upset any tests for sterility which may be applied to the substances without inactivation. For a successful test for sterility, the antibacterial properties should be destroyed as quickly as possible, certainly within an hour or two.

Penicillin is selectively antibacterial against Gram-positive organisms, and, in 1940, Abraham and Chain[79] prepared an extract of *E. coli* and of other Gram-negative organisms containing an enzyme, since called penicillinase, which was the responsible inactivating agent. The commercial enzymes, "taka-diastase" and "clarase", have been described as

penicillin inactivators and their use advocated in sterility tests for penicillin[80], but the active agent in these preparations is probably the same as the bacterial penicillinases.

It is important to use highly active preparations of penicillinase to reduce the time of inactivation of the penicillin, and one of the most satisfactory methods of preparation is to add penicillinase solution continuously by growing cultures of a strain of *B. subtilis* as described by Duthie[81]. Stability of the enzyme is of equal importance. Duthie claimed stability to heat at 100° C. for 20 minutes for this preparation, and Smith and Smith[82] described the preparation of an enzyme concentrate from a paracolon bacillus which was thermostable when dry, but completely unstable in solution. Manson and Pollock[83] and Manson, Pollock and Tridgell[84] have discussed the heat stabilities and other properties of penicillinase preparations from *B. subtilis* and *B. cereus*, finding considerable variations according to the source and method of preparation.

The United States Federal Register[85] prescribed hydroxylamine hydrochloride as the inactivator, used in a 1 in 300 solution. This is not a happy choice, however, and is to be discouraged, because of the known bacteriostatic properties of hydroxylamine. Even a 0·001 per cent. solution is said to be inhibitive[86]. Certain sulphydryl compounds can be used, but they are only effective in high concentration or when they are present in excess.

Inactivation of Streptomycin. Streptomycin and its salts are much more stable than is penicillin. It is claimed that cysteine[87], cevitamic acid[88], and carbonyl reagents[89] are effective inactivators. It has been shown, however[88], that several so-called "inactivating" agents, such as glucose, ketones and sulphydryl compounds may only be partially effective, and that some only reduce the activity of the streptomycin, which has optimum activity in slightly alkaline conditions, by rendering the medium acid. Cysteine is the most reliable of the inactivators suggested, its action being attributed to the blocking of an active grouping in the streptomycin molecule. Lipositol is said to inhibit streptomycin activity[90], but this has since been contradicted[91].

An interesting recent discovery is the substance produced by *Pseudomonas pyocyanea* first described by Sureau and others[92] and thought to be an enzyme. The substance has since been purified and crystallised by Bergman and others[93] and by Lightbown[94], and reported by the latter to be a mixture of several closely related derivatives of 4-hydroxyquinoline-*N*-oxide. It was claimed to have the property of inactivating streptomycin and also dihydrostreptomycin[91] and neomycin[93], and as such held out promise as suitable for use in sterility tests on these activities. Subsequent investigation has not fulfilled this promise[94], because the material itself is bacteriostatic and it only slightly antagonises the action of streptomycin against *E. coli*.

Inactivation of other Antibiotics. Apart from a suggestion of an inactivator for neomycin[93], there is no known antagonist against other antibiotics. In carrying out sterility tests, therefore, it is possible only

to use a reasonable dilution in broth of the sample. This means that an enormous volume of medium is required to test a reasonable amount of sample, or, the amount of sample tested is undesirably small. The tendency, as indicated in the B.P. test for sterility on aureomycin, is to accept the idea of using only a small sample. This cannot, however, be considered satisfactory.

Conclusions

The importance of carrying out adequate tests for sterility on preparations intended for injection or for topical application to open wounds cannot be over-estimated. Because of the anticipated low incidence of bacterial infection in the preparations, such tests must be made in media known to be capable of supporting the growth of small numbers of a variety of commonly occurring micro-organisms. They must include tests for aerobic and anaerobic bacteria growing at 37° C., and should also be capable of detecting moulds and psychrophilic bacteria; this can be done by setting up additional tests incubated at 22 to 25° C.

As well as checks on the nutritive properties of batches of media, control tests should also be made at intervals on the method of carrying out the tests, to ensure that the introduction of accidental contaminations is at a minimum.

Media used for aerobic tests can be made with peptone and meat extract or from the tryptic or papaic digestion of meat. Other sources of nutrients can be used, but confirmation of their ability to support growth of bacteria is essential. For anaerobic tests, nutrient broth with a layer of minced meat in the bottom and sealed with soft paraffin is satisfactory, but other reducing agents, in particular thioglycollic acid, have advantages over meat.

Adequate precautions must be taken to render any bacteriostatic substance in the injection inactive. In the case of phenolic substances simple dilution in the test broth is sufficient, but mercurial bacteriostatics require a more positive inactivator. Thioglycollic acid is the most satisfactory. In some cases the medicament is itself bacteriostatic or bactericidal, but this can generally be overcome by dilution in broth. The antibiotics, however, need special treatment. Penicillin can readily be inactivated by using a potent penicillinase preparation; streptomycin can be inactivated effectively by using cysteine in the medium. No inactivator is known for other antibiotics, and the position with them is, therefore, less satisfactory.

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