# A TELLURITE MEDIUM FOR THE IDENTIFICATION AND ISOLATION OF *CORYNEBACTERIUM DIPHTHERIAE*

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## INTRODUCTION

It appears to be almost generally acknowledged that the use of Loeffler's inspissated serum medium for the identification of *Corynebacterium diphtheriae* gives inferior results when compared with media containing potassium tellurite as a selective inhibitor. The following are some of the reasons for this inferiority:

(1) The method is dependent on the microscopical identification of an organism whose morphology is variable and in some cases indistinguishable from organisms which are not *C. diphtheriae*.

(2) Being dependent on microscopical examination the chances of overlooking small numbers of *C. diphtheriae* are considerable.

(3) Even when comparatively large numbers of *C. diphtheriae* are initially present in the pathological material under examination they may not be detected in cultures on Loeffler's serum medium if other organisms are also present which rapidly overgrow the *C. diphtheriae* in the absence of a selective inhibitory agent.

Whilst many existing media containing potassium tellurite are superior to Loeffler's serum medium it would appear that many laboratories, especially the smaller ones, do not use tellurite media. This may in part arise from the fact that it is not particularly easy to acquire confidence in the use of these media. The growing number of new and superseded tellurite media may perhaps be regarded as an indication that the media at present available are not altogether satisfactory.

It may be useful to set out what appear to be some of the main desirable characteristics of a tellurite medium:

(1) The growth of all strains of *C. diphtheriae* should be unmistakable after 18 hr. incubation at 37° C.

(2) It should be easy to differentiate between colonies of all strains of *C. diphtheriae* and other organisms, or the selectivity of the medium should be such that the growth of all other organisms, except *C. diphtheriae*, is suppressed. It is probable that the former is better if (1) is to be achieved.

(3) The differentiation of the types of *C. diphtheriae* from colonial appearance should be easy.

The medium, whose preparation and properties are to be described, is put forward as an attempt to fulfil the above conditions as far as possible. This medium has proved most satisfactory in routine use in the Public Health Laboratory at Hereford during the past six years and bears certain resemblances to the medium recently described by Tinsdale (1947).

## PREPARATION OF THE MEDIUM

Whilst the growth-factor requirements of *C. diphtheriae* have not, as yet, been completely determined for all strains, it is known that certain heat-labile substances are essential for the growth of this organism, and it is therefore important that the medium should be prepared exactly as described and only from the particular brand or type of material indicated, at the same time avoiding all unnecessarily prolonged heating and high temperatures.

The medium consists of an agar base to which are added tryptic casein digest, L-cystine, sheep serum, extract of sheep red blood corpuscles and potassium tellurite.

### (1) *Preparation of the agar base*

The following mixture is prepared:

| | |
|---|---|
| Lab Lemco | 3·0 g. |
| †Difco proteose peptone | 3·0 g. |
| ‡Sodium lactate (50 % solution) | 9·0 ml. |
| Pure glycerol | 3·0 ml. |
| Agar powder | 7·5 g. |
| Distilled water to 186 ml. | |

The reaction of the above mixture is adjusted as nearly as possible to pH 7·6 (once the amount of standard NaOH required has been determined it remains very nearly constant for subsequent batches) *before* autoclaving at 10 lb./sq.in. pressure for 15 min.

* The preliminary work was done in the Biochemical Department, School of Medicine, Leeds, and continued in the Bacteriology Department, Medical School, Birmingham. The final form was developed by the author when he was a member of the staff of the Emergency Public Health Laboratory Service in Shropshire and Herefordshire.

† Difco proteose peptone appears to be more suitable than any other kind of peptone which has been tried.

‡ Sodium lactate may be obtained as a 50 % solution or it can be prepared by neutralizing lactic acid with a 40 % solution of sodium hydroxide followed by dilution with distilled water to give a 50 % w/v solution.

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(2) *Preparation of the tryptic casein digest*

6·0 g. of anhydrous sodium carbonate and 2·0 g. of trypsin powder* are added to 100 g. of casein;† these are well mixed in the dry state before adding 1000 ml. of distilled water and 20 ml. of chloroform. The mixture is incubated at 37° C. for 3 days and is well shaken each day. After the final shaking the digest is allowed to stand for ½–1 min. before the supernatant liquid is poured off from the coarse residue and strained through a layer of cotton-wool supported by butter muslin. The digest is then heated to 60° C. for 30 min., and thereafter the reaction is adjusted to pH 7·2 (bromothymol blue). The digest is then stored in screw-cap medical flats in 500 ml. quantities with a little chloroform added as preservative. This digest appears to keep indefinitely in the dark at room temperature. After standing for some time at room temperature a flocculent precipitate separates; this is removed from the digest which is then ready for use. The digest appears to have a tendency to become somewhat more alkaline on standing, especially when freshly prepared, and therefore it is advisable to check the reaction every few weeks and if necessary adjust to pH 7·2.

(3) *Preparation of the* L-*cystine solution*

0·18 g. of L-cystine and 0·18 g. of anhydrous sodium carbonate are added to 6 ml. of sterile distilled water in a sterile 1 oz. screw-cap bottle. The suspension is heated with frequent shaking to a temperature not higher than 55° C. for a time not longer than is just necessary for the L-cystine to dissolve. The resulting solution appears to be self-sterile after 2 days in the refrigerator, in which it should be stored until required for use; this should be within a few days of preparation. Should the solution acquire a faint yellowish colour, either as a result of undue heating to promote solution of the cystine or of storage at room temperature, it tends to become increasingly inhibitory and should not be used. The complete medium will, however, keep for several weeks without any apparent deterioration if stored in the refrigerator. There may, in fact, be some slight improvement for a time, on keeping.

(4) *Preparation of sheep serum*

Normal sheep serum, not containing obvious haemoglobin, is maintained at a temperature of 56° C. for 2 hr. It is then distributed in small sterile screw-cap bottles and small quantities of chloroform added. It is then stored in the refrigerator after shaking well. Sheep serum appears to be by far the most effective of all easily obtainable sera and can apparently be kept for long periods without deterioration.

(5) *Preparation of extract of sheep red blood corpuscles*

Sheep blood is run directly into a 2 % solution of sodium citrate in the proportion of approximately 650 ml. of whole blood to 150 ml. of the citrate solution. The red blood corpuscles are separated by centrifuging. To 1000 ml. of the cells 100–150 ml. (the amount necessary varies and probably depends, amongst other things, on the speed and time of centrifuging) of sulphuric acid,‡ prepared by adding 6 ml. of 98 % Analar sulphuric acid to 94 ml. of distilled water, are added with vigorous and rapid stirring to ensure that the sulphuric acid is evenly distributed before the mixture sets to a rubbery mass. This is allowed to stand at room temperature for 2–3 days, and the resulting dark brown mass is then broken up and kneaded with distilled water (1500 ml. of distilled water to the material obtained from 1000 ml. of sheep red blood corpuscles) until a suspension is obtained. After standing for a few hours, with occasional gentle shaking by hand, the bulky suspended particles are easily separated from the extract by squeezing through several thicknesses of fine butter muslin. The extract is finally cleared of the small remaining amount of suspended matter by centrifuging and should then be a pale brown colour. When one drop of the extract is added to approximately 1 ml. of a saturated solution of phenol in water a voluminous white precipitate should be produced. The reaction of the extract should be adjusted to pH 6·8 (bromothymol blue), at no stage should it exceed pH 6·8, and any slight precipitate removed by centrifuging. The extract should now be distributed in small sterile screw-cap bottles, with a little chloroform added as preservative, and stored in the refrigerator, where it appears to keep indefinitely. In the course of time small traces of reddish brown blood pigment may be precipitated, these should be removed by centrifuging. The extract can be used before this precipitation takes place and as soon as any contaminating organisms have been killed by the chloroform.

(6) *Potassium tellurite solution*

This is prepared as a 1 % solution in distilled water. It appears to keep indefinitely when stored in the

* Trypsin powder supplied by British Drug Houses Ltd. is used.

† It appears to be important to use the casein known as Light White Soluble as supplied by British Drug Houses Ltd.; other types of casein have not proved satisfactory for this purpose.

‡ Hydrochloric acid should not be substituted for sulphuric acid, since it has been found that when hydrochloric acid is used the constituents of the mixture cannot be separated readily.

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refrigerator in screw-cap bottles and is self-sterile after 2 days.

It should be noted that, with the exception of the L-cystine solution, all these constituents can be prepared in bulk for use over a considerable period.

### *Addition of the other components to the agar base*

The agar base is removed from the autoclave as soon as possible after sterilization; 60 ml. of tryptic casein digest are added to the agar base, and after shaking to ensure thorough mixing the whole is cooled to about 55° C. and 6 ml. of L-cystine solution, 15 ml. of sheep serum, 30 ml. of the extract of sheep red blood corpuscles and 3 ml. of the potassium tellurite solution are added. It is important to add each solution in the order indicated and to mix well by rotation of the flask after the addition of each component. It will be found convenient to keep the flask containing the agar wrapped in some heat-insulating material after cooling to 55°C. The mixture is finally distributed between *not more than* twenty sterile 4 in. Petri dishes by pouring and not by pipetting.

## GENERAL PROPERTIES OF THE MEDIUM

Since the medium does not contain blood or other pigments it is almost as clear and transparent as nutrient agar. This characteristic greatly facilitates the examination of any bacterial colonies appearing during the course of incubation. The growth of all the many strains of *C. diphtheriae* examined is rapid and is characterized by the production, after some 15 hr. incubation, of a deep brownish discoloration in the medium around each colony. With two exceptions, which will be described later, no other organism, among the many so far examined, has produced this effect within the first 18–24 hr. incubation; its production by the colonies of *C. diphtheriae* appears to be dependent on the presence of L-cystine in the medium and possibly due to reduction of the L-cystine by some metabolic product or enzyme system of the diphtheria bacilli, since it is not produced in the medium in the absence of L-cystine and there is no change of colour when L-cystine and potassium tellurite are heated together in solution unless a reducing agent is present.

The concentration of potassium tellurite used (0·01 %) is much lower than the concentration generally used (0·04 %) to achieve adequate selectivity and which is also said to inhibit the growth of some *mitis* strains of *C. diphtheriae* (Knox, 1944).

The use of serum and an extract of red blood corpuscles from which blood pigments have been removed yields a medium which besides being uncoloured appears to retain the growth factors of whole blood and at the same time eliminates the possible inhibition of the growth of some strains of *C. diphtheriae* by blood pigments or their derivatives (Glass, 1939).

## COLONIAL APPEARANCE OF THE TYPES OF *CORYNEBACTERIUM DIPHTHERIAE* AND OTHER ORGANISMS ON THE MEDIUM

Whilst old stock cultures of *C. diphtheriae* yield satisfactory growth on the medium the growth of strains freshly isolated from pathological material is usually better.

### (*a*) *General account of the characterization of* Corynebacterium diphtheriae *on the medium*

All the numerous strains of *C. diphtheriae* so far examined, from many different localities, produce a characteristic brown discoloration in the medium around each colony after 15–18 hr. incubation. These discoloured zones are always quite transparent and without any trace of iridescence. In the case of very widely spaced colonies, e.g. those arising from swabs from convalescents, the discoloration may only appear after 24–30 hr. incubation. Only two other organisms have been encountered which produce a brownish discoloration within 24 hr. One is a coccus resembling *Micrococcus tetragenus* which has occurred on two occasions only. The discoloration produced in the medium by this organism is foxy red and easily distinguished from the deep brown discoloration produced by *Corynebacterium diphtheriae*. The author is most grateful to Dr R. E. Jones of the Public Health Laboratory Service for drawing his attention to the other organism which has been observed to produce the discoloration effect within 24 hr. This organism has the morphology of a diphtheroid and has been isolated from cow's milk and from swabs from cases of tonsillitis. It produces acid in the presence of starch, glucose and maltose and liquefies gelatin slightly. It is pathogenic for guinea-pigs, and diphtheria antitoxin does not protect the animals against this organism. In spite of its capacity to ferment starch the organism has the colonial appearance of a *mitis* strain of *C. diphtheriae* on tellurite media. This organism appears to be similar to the anomalous members of the *Corynebacterium* group described in the *Monthly Bulletin of the Emergency Public Health Laboratory Service* for February 1942.

The broad distinguishing features of the three types of *C. diphtheriae* as set out below appear to be constant, but minor variations of colonial appearance within a type are not unusual.

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(1) *Corynebacterium diphtheriae mitis*

Occurs as smooth, moist, convex colonies which smear when pushed with a needle. The colonies are variable in size, growing to a diameter of 1–2 mm.; they have a central zone which by reflected light is dark greyish brown in colour when young, becoming greyish blue with age, and have a light brown periphery. As the colony increases in size and age the peripheral zone widens leaving a small sharply defined black central zone, especially when observed by transmitted light. The brown discoloration produced in the medium is very pronounced even after 15–18 hr. incubation, and the colonies must be well separated to show individual zones of discoloration, although separate colonies are not necessary for identification. After 35–40 hr. incubation the surface of the colony is very moist and shiny; there may be a central papilla, but radial striations and peripheral indentations are not developed.

(2) *Corynebacterium diphtheriae gravis*

The colonies have a matt surface, usually have a small central papilla, and can be pushed in front of a needle without smearing. The colonies are variable in size up to a diameter of 2–3 mm. They are greyish brown by reflected light when young, becoming a pale greyish blue, lighter in colour than the *mitis* colonies, as age and size increases. After 30–40 hr. incubation the colony, when seen by transmitted light, has a small black central part surrounded by an almost colourless peripheral zone which shows indentations and radial striations. The brown discoloration in the medium around the colonies is quite marked even after 15–18 hr. incubation, although it is perhaps not quite so intense as with the *mitis* strains. As in the case of *mitis* strains the colonies must be well separated to show individual zones of discoloration and here also separation of the colonies is not necessary for identification.

(3) *Corynebacterium diphtheriae intermedius*

The colonies are characteristically uniform in size and smaller than those of either the *gravis* or the *mitis* strains. Colonial size varies from strain to strain but rarely reaches 1·5 mm. in diameter and is usually 0·75–1·0 mm. The colonies often have a central papilla and slightly raised margins. After 18 hr. incubation the colonies are dark greyish brown in colour with a narrow, paler, peripheral zone. Again there is a dark brownish discoloration in the medium around the colony. In many cases the colonies of *intermedius* strains appear to have a narrow zone of unstained medium between the periphery of the colony and the zone of brown discoloration. Unlike the colonies of *mitis* and *gravis* strains, which invariably become lighter in colour with age, the *intermedius* colonies remain dark brownish black; this appears to be an important distinguishing characteristic. The dark portion of the colony covers the whole or a very large part of the colony, depending on whether the colonies are widely separated or relatively close together.

(*b*) *Corynebacterium hoffmani*

The colonies of this organism are uniform in size, about 1 mm. in diameter, with smooth hemispherical surfaces which do not become as moist as those of *mitis* strains of *C. diphtheriae* and which remain devoid of papillae or radial striations even after 40 hr. incubation. They are whitish in colour after 18–24 hr. incubation. After 30–40 hr. incubation they have a central steel grey zone with a lighter periphery. There is no brown discoloration of the medium except in the case of a few strains that produce only a feeble browning, which occurs, however, only after 30–40 hr. incubation and only on plates where the colonies are crowded together. Such discoloration can be differentiated from the discoloration produced by strains of *C. diphtheriae,* since the boundary of discoloration produced by a group of *C. hoffmani* colonies does not extend beyond the group, whereas the discoloration around all *C. diphtheriae* colonies extends well beyond the outer colonies of a group and is present around each colony no matter how well isolated.

(*c*) *Corynebacterium xerosis*

The colonies are large, flat, moist and almost black after 18 hr. incubation with no discoloration of the medium around the colonies. After 30–40 hr. incubation there has been some increase in size of the colony, and the colour has become very light blue grey but there is no discoloration of the medium around the colonies.

(*d*) *Slow-growing diphtheroids*

Many types having very different colonial appearances compared with the types of *C. diphtheriae* have been observed. They usually occur as small, often irregularly shaped or umbilicate, almost black colonies. None of these organisms appears to produce a discoloration in the medium around the colonies until after 30–40 hr. incubation. Only a small number of these diphtheroids have been observed to produce a brown discoloration in the medium after 30–40 hr. incubation; even then it is comparatively feeble, and instead of being transparent as in the case of *C. diphtheriae* it has an iridescent appearance as of minute, irregularly arranged, metallic crystals in the medium. This discoloration is quite different in appearance when compared with the discoloration produced by colonies of *C. diphtheriae.*

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### (*e*) *Staphylococci and streptococci*

These organisms produce large and small black colonies respectively. Discoloration of the medium around the colonies has not been observed with any strain of these organisms.

### (*f*) *Yeasts*

These organisms produce whitish colonies after 18 hr. incubation. These colonies become brown on further incubation, but none has been observed to produce a discoloration in the medium around the colony.

### (*g*) *Large Gram-negative rods and Gram-negative cocci*

These organisms do not appear to be able to grow on the medium.

The differences in colonial appearances on this medium between the strains of the three types of *C. diphtheriae*, diphtheroids and other organisms is much greater than can be indicated in written descriptions.

## RECOMMENDATIONS FOR THE INOCULATION OF THE MEDIUM WITH PATHOLOGICAL MATERIAL

It has been found practicable to inoculate from four to six throat swabs from suspected cases of diphtheria on to each 4 in. plate of the medium by merely rubbing the swab over an area delineated on the outside of the plate.

It would appear that nasal swabs are best inoculated on to a quarter or half of a 4 in. plate by rubbing the swab over a small part of the area to be inoculated and then using a spreader to distribute the inoculum over the remainder of the area. This also appears to be necessary to detect small numbers of *C. diphtheriae* occurring amongst large numbers of *C. hoffmani* or *C. xerosis* in swabs from convalescent cases of diphtheria and carriers.

Aural swabs appear to give the best results when the inoculum is spread over half or the whole of a 4 in. plate.

## SUMMARY

1. The preparation and characteristics of a tellurite medium, containing, in addition to a peptone and Lemco agar base, a tryptic hydrolysate of casein, sodium lactate, L-cystine, sheep serum, an extract of sheep red blood corpuscles and 0·01 % potassium tellurite, is described for use in the identification and isolation of *Corynebacterium diphtheriae*.

2. The colonial appearances of the three types of *C. diphtheriae* on the medium are described, and the distinguishing features between these and the colonial appearances of diphtheroids and other organisms liable to occur on pathological material are indicated.

3. Recommendations are made concerning what has so far proved to be the most suitable manner of inoculation of pathological material on to the medium.

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