# DIFFERENTIAL MEDIA FOR RECOGNITION OF *B. DIPHTHERIAE* AND ASSOCIATED ORGANISMS.

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Certain difficulties attendant upon bacteriological diagnosis recur constantly in connection with the notification, isolation, and discharge of patients suffering from diphtheria, and from every point of view it becomes increasingly necessary to seek means to minimise, as far as possible, the number of 'missed' and 'carrier' cases.

The ready bacterioscopic recognition of certain organisms prone to infect the upper respiratory tract is by no means sure, and, more especially with diphtheria, negative results are often misleading.

Particularly with subcultures of suspected material on media the basis of which is animal blood-serum, failure to recover the diphtheria bacillus is often due to overgrowth of associated organisms: occasionally the difficulty may be explained away by the invocation of latency, in that the transplanted organism does not take kindly at first to its new home. The comparatively rapid growth of *B. subtilis*, *B. megatherium*, *B. Hofmanni*, and of certain *Torulae* or *Micrococci* is the usual source of trouble.

From sowings of an admixture of *B. diphtheriae* and *B. subtilis* or *B. megatherium* I have often been able to recover the former from a surface growth on coagulated blood-serum by scraping along the upper dried semi-hornified portion of the serum precisely at that point or margin where the culture of *B. subtilis* or *B. megatherium* ceased to spread: it is a matter of greater difficulty to recover *B. Hofmanni* when present in place of *B. diphtheriae* in like mixtures.

Whereas the condition of dryness in a serum culture is unfavourable for the development of *B. subtilis* and *B. megatherium* it favours the growth of *B. diphtheriae*, a fact which may, perhaps, be explained by the close relationship of the latter with the myco-bacteria.

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## I. AGAR MEDIA.

*Dryness of Medium.* For practical purposes 'dryness' of a medium may be induced by the addition of salts, such as chloride of calcium, or by the addition of substances such as glucose or glycerine.

For convenience in rapid working nutrient agar was used as a basis in place of blood-serum, especially in the earlier series of experiments to be described. Surface streaks on plates were made and the cultures incubated at 37° C. with the following results:—

(1) (*a*) $CaCl_2$ 5 % added: *B. diphtheriae* and *Staphylococci* plentiful growth; *B. subtilis*, slight growth; *B. Hofmanni*, *B. megatherium* and *B. coli* trace only.

(*b*) $CaCl_2$ 2½ % added: *B. megatherium* and *B. coli* alone markedly restrained; some restraint of *B. Hofmanni*.

(2) Glucose 10 % added: *B. diphtheriae*, *B. subtilis* and *B. Hofmanni* apparently not restrained. Growth of *B. coli* and *B. megatherium* appears to be inhibited.

*Action of Salts of Saliva and Bile.* Neither glucose nor calcium chloride alone or in conjunction would appear to render a medium specially favourable to *B. diphtheriae* while excluding the other organisms already specified. The scope of the experiments was therefore extended so as to determine the action of certain salts of the saliva and bile, potassium sulphocyanide and taurocholate of soda respectively, both in agar and coagulated blood-serum media. In addition, both ammonium and sodium sulphocyanide have been tested, and, as an indicator to determine the reaction of the medium, 1 % of a 1 % watery-neutral red has usually been added. Fuchsin, decolorised by alkali, has on occasion been tested as an indicator, but there is little advantage attending its use.

(3) The addition of ammonium sulphocyanide to agar medium already containing glucose inhibited the growth of the following organisms in the order:

(*a*) *B. megatherium*, (*b*) *B. coli*, (*c*) *B. diphtheriae*, (*d*) *B. subtilis*, (*e*) *B. Hofmanni*, which is least affected, not being, apparently, unrestrained even in presence of 2½ % AmCNS.

(4) AmCNS, in the presence of $CaCl_2$, continued to inhibit especially the growth of *B. Hofmanni*.

(5) ½ % bile salt, in presence of 5 % $CaCl_2$, appeared to inhibit greatly the growth of all the organisms considered.

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(6) $\frac{1}{2}$% bile salt, in presence of glucose and ammonium sulphocyanide, favoured especially the growth of *B. Hofmanni*, and, with neutral-red, a purple-red growth results surrounded by a clear zone.

The foregoing series of tests were carried out by way of a preliminary trial. In the following series all the media were composed of nutrient agar to which neutral-red has been added as specified, together with certain salts. The cultures were incubated at 37° C.

(A) *Potassium Sulphocyanide* was added in quantities varying from $\frac{1}{8}$% to $2\frac{1}{2}$%—with the result that $\frac{1}{4}$% and over inhibited the

*A Series.*

Colour denotes appearance of colonies: T. L. = transmitted light, R. L. = reflected light.

| | | *B. diphtheriae* | *B. subtilis* | *B. coli* | *B. megatherium* | *B. Hofmanni* | Mixture of *Staphylococci* and *B. Hofmanni* |
|---|---|---|---|---|---|---|---|
| Control, neutral red agar plate KCNS omitted | T. L. | Faint pink | Salmon pink | Bluish pink | Bluish fluorescence | Bluish pink | Brick red |
| | R. L. | Pinkish | Pinkish | Pinkish | Pinkish | Pinkish | Pinkish |
| Amount of growth | | 3* | 3 | 3 | 2 | 3 | 3 |
| $\frac{1}{8}$% KCNS added | T. L. | Pink | Yellow red | Brick red | Cream | Yellow | Yellow |
| | R. L. | Claret red | Yellow red | Yellowish | Cream | Yellow | Yellow |
| | Growth | 3 | 3 | 2 | 3 | 3 | 3 |
| $\frac{1}{4}$% KCNS added | T. L. | Pink | Yellow red | Brick yellow red | Cream | Yellow | Brick yellow |
| | R. L. | Red | Yellow red | Yellow | Cream | Yellow | Yellow |
| | Growth | 3 | 3 | 3 | 1 | 3 | 3 |
| $\frac{1}{2}$% KCNS added | T. L. | Pink | Yellow red | Brick yellow red | — | Brick yellow | Yellow |
| | R. L. | Claret red | Brick | Yellow | — | Yellow | Yellow |
| | Growth | 3 | 3 | 3 | $\frac{1}{2}$ | 3 | 3 |
| 1% KCNS added | T. L. | Claret red | Brick red | Pink | Very faint pink | Yellow red | Yellow red |
| | R. L. | Claret red | Brownish | Yellow red | — | Yellow red | Yellow red |
| | Growth | 2 | 3 | 1 | $\frac{1}{4}$ | 3 | 3 |
| $2\frac{1}{2}$% KCNS added | T. L. | Reddish | Brick | —? | —? | Red | Brick red |
| | R. L. | Deep pink | Brick | —? | —? | Deep pink | Deep pink |
| | Growth | 2 | 3 | ? | ? | 2 | 3 |

* The numbers $\frac{1}{2}$, 1, 2, 3, 4 in the tables indicate progressive degrees of growth measured objectively.

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growth of *B. megatherium*, and 1 % and over inhibited that of *B. coli*. Where KCNS is present to the amount of $2\frac{1}{2}$ % or less, the naked-eye appearance of colonies of *B. diphtheriae* both by transmitted and reflected light was sufficiently distinctive; above $2\frac{1}{2}$ % the tints developed by the colonies were not sufficiently distinctive to be of value.

(B) *Bile Salt* (taurocholate of soda-ox-bile) was added in quantities varying from $\frac{1}{8}$ % to 5 % to neutral-red agar and plates poured. Prolonged incubation at 37° C. Results: with $\frac{1}{4}$ % there is some restraint and with $\frac{1}{2}$ % complete inhibition of growth of *B. subtilis* and *B. megatherium*. The addition of $\frac{1}{4}$ % to 1 % shows up special coloration of colonies of *B. diphtheriae* when present in pure culture.

*B Series.*

Control—see A series.

| | | *B. diphtheriae* | *B. subtilis* | *B. coli* | *B. megatherium* | *B. Hofmanni* | Mixture of *Staphylococci* and *B. Hofmanni* |
|---|---|---|---|---|---|---|---|
| $\frac{1}{8}$ % Bile salt added | T. L. | Yellow | Bluish fluoresc. | Bluish fluoresc. | Bluish fluoresc. | Bluish yellow | Bluish yellow |
| | R. L. | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow |
| | Growth | 3 | 2 | 4 | 2 | 3 | 3 |
| $\frac{1}{4}$ % | T. L. | Red yellow | Yellow | Yellowish-blue fluoresc. | Bluish fluoresc. | Bluish Yellow | Yellow |
| | R. L. | Red | Red | Red | Red | Red | Red |
| | Growth | 3 | 1 | 4 | Trace | 3 | 3 |
| $\frac{1}{2}$ % | T. L. | Red | — | Yellow fluoresc. | — | Yellow | Reddish pink |
| | R. L. | Red | — | Yellow fluoresc. | — | Yellow | Reddish pink |
| | Growth | 2 | Trace | 4 | 0 | 4 | 4 |
| 1 % | T. L. | Red | — | Yellowish-blue fluoresc. | — | Yellowish blue | Yellowish pink |
| | R. L. | Red | — | Yellow fluoresc. | — | Yellow | Yellowish pink |
| | Growth | 2 | 0 | 4 | 0 | 4 | 3 |
| 5 % | T. L. | Red | — | Red | — | Yellow centre, red periphery | Red |
| | R. L. | Almost black | — | Red | — | Yellow centre, red periphery | Red |
| | Growth | 2 | 0 | 2 | 0 | 3 | 3 |

(C) *Calcium Chloride* added to neutral-red agar plates. The effect of increasing the percentage of $CaCl_2$ in agar was to restrain the growth of all the organisms which were cultivated upon the

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medium. The presence of 1 % to 2½ % of this salt allowed *B. diphtheriae* and certain *Micrococci* to grow freely while inhibiting markedly the growth of *B. subtilis*, *B. coli*, *B. Hofmanni*, and *B. megatherium*. With 1 % $CaCl_2$ there appeared a distinct clear zone around the red growth of cocci, while throughout the medium generally there was a faint haze.

*C Series.*

| | | *B. diphtheriae* | *B. subtilis* | *B. coli* | *B. megatherium* | *B. Hofmanni* | Mixture of *Staphylococci* and *B. Hofmanni* |
|---|---|---|---|---|---|---|---|
| Control. No $CaCl_2$ added | R. L. | Pink | Pink | Faint pink | Pink | Pink | Red |
| | T. L. | Red | Bluish pink | Bluish fluoresc. | Bluish fluoresc. | Bluish fluoresc. | Yellow |
| | Growth | 3 | 3 | 3 | 3 | 3 | 4 |
| 1 % $CaCl_2$ | R. L. | Faint red | Pink | ? growth | Very faint pink | Faint pink | Clear zone around red growth |
| | T. L. | Faint red | Pink | ? growth | — | — | Ditto |
| | Growth | 2 | 2 | ½ | ¼ | 2 | 3 [a] |
| 2½ % $CaCl_2$ | T. L. | Pink | Pink | — | — | — | Reddish pink |
| | R. L. | Pink | — | — | — | — | Reddish pink |
| | Growth | 3 | 1 | ¼ | ¼ | ½ | 3 [b] |
| 2½ % $CaCl_2$. Another series of strains of organisms used | | 1 | 1 | 0 | 0 | Trace | — |
| 5 % $CaCl_2$ | T. L. | Pink | Pink | — | — | Pink | Pink [c] |
| | R. L. | — | — | — | — | — | — |
| | Growth | 1 | 1 | ¼ | ¼ | 1 | 1 |
| 7½ % $CaCl_2$ | ... | Trace | Faint trace | 0 | 0 | Trace | Faint trace |

[a] 1 % $CaCl_2$—growth: nearly all cocci. [b] 2½ % $CaCl_2$—practically all cocci. [c] 5 % $CaCl_2$—all cocci. Thus the addition of $CaCl_2$ would appear to inhibit the growth of *B. Hofmanni* in a mixed culture containing *Staphylococci*, *B. Hofmanni* and *B. diphtheriae*, a result which was confirmed by subsequent experiment.

(E), (O) and (I). In addition to potassium sulphocyanide various other cyanides—*potassium cyanide* (= E Series), *potassium ferrocyanide* (= O Series), and *potassium ferricyanide* (= I Series)—were added in varying percentages to neutral-red agar plates. Surface streak cultures were made and incubated as before.

*Tints*: R = Reddish, W = Whitish, Y = Yellow, Br = Brownish.

The numbers ½, 1, 2, 3 denote progressive degrees of growth.

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*E Series.*

| | *B. diphtheriae* | *B. subtilis* | *B. coli* | *B. megatherium* | *B. Hofmanni* | Mixture of *cocci* and *B. Hofmanni* |
|---|---|---|---|---|---|---|
| KCN % added, ¼ | R 3 | W 3 | W 1 | R 2 | Y 2 | Y 3 |
| ½ | R 1 | W 2 | 0 | P 2 | Br 2 | Br 3 |
| 1 | R 3 | W 3 | W ½ | Y 2 | Y 2 | Y 3 |
| 2½ | 0 | W 3 | 0 | W 3 | 0 | W 3 |

Much inconvenience is experienced in working with media to which potassium cyanide is added owing to the constant liberation of prussic acid whilst pouring the plate and during period of incubation.

Owing to this continual discharge of HCN vapour the medium tends to become increasingly alkaline and yellow as incubation proceeds, and therefore at the moment of pouring the plates the reaction of the medium should be adjusted with free HCl so as to indicate faint acidity.

In general *B. diphtheriae* gives a reddish coloration which is slightly distinctive and serves to differentiate its colonies from those of the remaining organisms with the exception of *B. megatherium.* The colours fade rapidly changing to pale yellow with the development of increased alkalinity of the medium.

(O) *Potassium Ferrocyanide.*

| | *B. diphtheriae* | *B. subtilis* | *B. coli* | *B. megatherium* | *B. Hofmanni* | Mixture of *Staphylococci* and *B. Hofmanni* |
|---|---|---|---|---|---|---|
| % $K_4FeCy_6$ added, ¼ | R 3 | W 3 | W 2 | R 3 | YBr 3 | R 3 |
| ½ | R 3 | W 2 | 0 | PR 3 | BrR 2 | R 3 |
| 1 | 0 | W 3 | 0 | PR 3 | BrR 3 | R 3 |
| 2½ | 0 | W 3 | 0 | W 3 | YR 3 | R 3 |

Thus the salt in quantities exceeding ¼ % serves to inhibit *B. coli.* *B. diphtheriae* is restrained when the quantity of salt present exceeds ½ % and, in any case, the coloration of its colonies is never sufficiently distinctive.

(R) *Boric Acid* added to agar in quantities up to 2 % allows the growth of *B. diphtheriae, Staphylococci, B. Hofmanni* and certain *Streptococci* to take place but it inhibits markedly growth of *B. subtilis* and *B. megatherium.* Thus with a mixture of *Streptococci, Staphylococci* and *B. subtilis* (subcultured from an abscess on plain nutrient agar), the proportion of *B. subtilis* to other organisms was over 1000 to 1 and it was with difficulty that the *Streptococci* and *Staphylococci* were recognised. Subcultivation at 37° C. on agar containing 2 % boric acid

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showed an approximately equal distribution of *B. subtilis* and the various forms of cocci. On making further subcultures, on agar containing 4 % boric acid, the difficulty was reversed, *B. subtilis* becoming hard to find, the culture appearing to be a mixture of *Staphylococci* and *Streptococci* only.

*Effect of Combined Salts.* As a result of the foregoing experiments bile salt 1 % (= B), potassium sulphocyanide $\frac{1}{2}$ % (= A), potassium cyanide $\frac{1}{4}$ % (= E), and potassium ferrocyanide $\frac{1}{4}$ % (= O), and glucose $\frac{1}{2}$ % (= g) were added in various combinations to neutral-red agar. Where KCN (= E) was added special care was taken to neutralise the alkalinity by the addition of HCl. The medium was plated and surface streak cultures made and incubated at 37° C.

*Combinations tested*: BE, BEg; BA, BAg; BEO, BEOg; BO, BOg; BOA, BOAE.

Organisms sown: *B. diphtheriae, B. Hofmanni, B. megatherium, B. subtilis, Micrococci, Torulae,* and *B. coli.*

*Results*: Bile salt (B) inhibited greatly the growth of *B. subtilis* and *B. megatherium.* Potassium cyanide (E), except when combined with glucose (g), inhibited for a time the free development of *Torula* which, on the medium containing the combination BEOg, gave deep red colonies.

Where *B. diphtheriae* was present the combinations BO, BAO; BEOg and BEO, BAOg, BOg gave red colonies when incubated at 37° C. for 24 hours.

The rapidity of acid production by the several media is in the order mentioned.

At 20° C. for 72 hours the coloration of colonies changed to brownish-yellow. With BO and BAO combinations acid production by the colonies of *B. diphtheriae* occurred in 12–18 hours, much earlier than with any of the remaining organisms tested, and, as a result of making surface streak inoculations of various artificial test mixtures of organisms on media containing BO or BAO combinations of salts and incubating at 37° C., it appeared that wherever reddish colonies developed in 12 hours *B. diphtheriae* was invariably found to be present. The absence of red colonies, however, was not evidence of the absence of *B. diphtheriae,* and it became necessary to continue the experiments.

(I) Thereupon *Potassium Ferricyanide* $\frac{1}{4}$ % (= I) and lactose 1 % (= l) were added as variants to the combinations of neutral-red agar already cited. Bile salt (= B) was constantly present in each combination.

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*Combinations tested*: BO, BI; BOg, BIg; BOE, BIE; BAO, BAI; BAOg, BAIg; BAOE, BAIE; BOgl, BIgl; BAOgl, BAIgl; BEO, BEI; BEOg, BEIg; BEOl, BEIl.

Organisms tested (surface streak cultures incubated at 37° C.): *B. coli*; mixture of *Torulae* and *B. Hofmanni*; mixture of *Cocci* and *B. Hofmanni*, and *B. diphtheriae*.

The following points were noted:

(1) The strain of *B. diphtheriae* employed did not grow on any of these combinations by the 88th hour.

(2) A large number of involution forms of Hofmann's bacillus were observed in subcultures during the early period of incubation.

(3) Whenever a medium contained both the salts AI *Torulae* alone flourished on incubation, all the remaining organisms tested—*B. coli*, *B. Hofmanni* and *Cocci*—being rigidly inhibited.

(4) Later, at 20° C., the growth of *Torulae* on media containing any of the following combinations: BIg, BAIg, BIgl, gave rise to colonies having a deep prussian-blue coloration in centre with reddish periphery. On examination with the lower power (× 60) crystals resembling calcium oxalate were observed associated with a dark amorphous deposit in the immediate neighbourhood of the growth.

(5) Rapidity of production of alkalinity by colonies of the various organisms tested depended on the combination of salts added to the media. The following was the order of appearance of alkalinity in the combinations tested: (*a*) BEI. (*b*) BEO. (*c*) BAO or BAI and lastly (*d*) BO or BI.

The foregoing combinations were repeated, save that the percentage of salts added was reduced by one half in each case, with the result that in those combinations from which bile salt had been excluded *B. diphtheriae*, when present, again flourished, giving rise to colonies of varying tints, the combinations I, IA, and IO yielding distinctive pinkish colonies. The proportion of I (*i.e.* potassium ferricyanide) added varied from $\frac{1}{8}$% to $\frac{1}{16}$%.

These results have not been regarded as sufficiently satisfactory to warrant the use of media containing any of the foregoing combinations of salts for the routine diagnosis of *B. diphtheriae*, and in place of agar sheep's blood-serum has been used.



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## II. Coagulated Serum Media.

Recently clotted sheep's blood was deprived of the exuded serum which then was placed in a refrigerator and allowed to sediment for three days. The clear supernatant straw-coloured fluid was at length carefully drawn off clear of the deposit of red blood corpuscles, for it is essential that the serum be free of such corpuscles before use.

To this serum there is added 25 % nutrient broth, 1 % glucose, and 1 % of 1 % watery neutral-red as indicator.

(Where a refrigerator is not available 2 % chloroform may be added to the serum which is then placed in a tall glass cylinder and sedimentation allowed to proceed at air temperature. The subsequent process of sterilisation serves to expel the added chloroform.)

To this serum were added various concentrations of salts as with agar, and the reaction adjusted to faint alkalinity so that on coagulation the medium has a yellowish-buff appearance.

*Bile Salt; Potassium Cyanide.* It quickly became apparent that the addition of either of these two salts to serum formed a useless mixture, for a substance was produced which either did not coagulate on heating, or, if coagulated, became soft and jelly like and finally liquefied in the process of sterilisation. These two salts were therefore discarded.

*Test Organisms: B. diphtheriae, B. Hofmanni, B. megatherium, B. subtilis, Staphylococcus pyogenes aureus,* other *Staphylococci, Streptococci,* and *Torulae,* all recently isolated from cultures derived from throat swabs.

Nomenclature as with agar media generally, *e.g.*

'A' = 1 % KCNS added to medium,

'R' = 1 %, R 2 = 2 %, R $\frac{1}{4}$ = $\frac{1}{4}$ %, R $\frac{1}{8}$ = $\frac{1}{8}$ % respectively, boric acid added.

'I' = 1 %, I $\frac{1}{2}$ = $\frac{1}{2}$ % respectively of potassium ferricyanide added.

On media containing combinations AR $\frac{1}{8}$ and AR $\frac{1}{2}$, colonies of *B. diphtheriae* gave distinctive pinkish growth as compared with a yellowish or yellow-brown growth given by the remaining test organisms. Similarly with medium containing I $\frac{1}{2}$, colonies of *B. diphtheriae* showed a distinctive pink coloration. Coagulated serum to which potassium ferricyanide has been added is, however, somewhat soft and friable and easily torn with slight pressure.

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At this stage it became apparent that mere acid production with specific coloration, pink in the case of neutral-red media, is not the sole desideratum to be fulfilled by a method to be used for the ready recognition of the presence of the diphtheria bacillus either in pure culture or admixed with other organisms. Rather a large number of varieties or strains of *Staphylococci* derived from throat cultures give rise, on incubation, to acid in the presence of glucose, and the problem becomes narrowed down to the evolution of a medium which, while specifically favourable to the growth of *B. diphtheriae*, will on incubation produce specific changes other than colour-change due to mere alteration in reaction.

A medium obtained by adding to neutral-red-broth-glucose-serum 1 % of potassium sulphocyanide and thereafter coagulated presents, with regard to the following organisms, certain phenomena which become noticeable on incubation of surface streak cultures at blood-heat. (This particular combination is hereafter referred to as the 'A' medium.)

(*a*) *B. diphtheriae:* Acid production in the first 18 hours. Growth not raised, colonies discrete, very small, barely distinguishable. The surface of the growth more nearly resembles ground glass in dryness and general appearance. The colonies are pink. The medium becomes pink and a bluish-pink tint diffuses out into the medium far beyond the immediate limits of the colonies. These phenomena are particularly well marked with subcultures taken direct from the throat or nose.

The subsequent behaviour of the culture at 20° C. is of interest in that the pink tint continues to diffuse throughout the medium and there appears to be a slight diminution of intensity of colouring in the immediate neighbourhood of the growth.

With subcultures taken from laboratory strains which have for many generations been propagated solely in artificial culture the foregoing phenomena—reddish coloration of colonies followed by specific coloration of medium with diffusion of tint throughout the medium—are not so intense.

(*b*) *Staphylococcus pyogenes aureus*: In 18 hours localised growth; colonies raised, tinted, distinguishable, discrete; acid production variable and depending entirely upon the strain employed. If there be marked acid production by the colonies, a pink colour develops in the medium. This coloration is usually confined to the immediate neighbourhood of the colonies. With some varieties of *Staphylococci*, however, diffusion takes place into the surrounding medium, and, in such cases, the diffusing



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tint is usually patchy or streaky in distribution. The bluish tint so characteristic of *B. diphtheriae* is absent, nor with colonies of *Staphylococci* does diffusion of tint occur so readily into the surrounding medium at 20° C.

(*c*) *Torulae* in 18 hours: Acid production variable, growth raised, usually creamy. Medium may be tinted pink and if diffusion occurs it is but slight and of a faint pink tint.

(*d*) *B. megatherium* in 18 hours: Creamy growth, browning of medium in neighbourhood of colonies.

(*e*) *B. subtilis* in pure culture in 18 hours: Easily recognisable crinkly growth—membranous. Acid production, if marked, gives rise to a brownish-pink coloration and if diffusion occurs the coloration is pink. There is no trace of blueness. With continued incubation there may be liquefaction of the medium.

During the first 18 hours' incubation of a mixture of organisms containing either *Staphylococci* or *B. diphtheriae* in addition to *B. subtilis* there is some inhibition of growth of *B. subtilis* and apparently little restraint of growth of the remaining organisms. If incubation be continued for another 24 hours however, there is a marked acceleration of growth of *B. subtilis* giving rise to the usual membranous appearance as seen with the pure culture of that organism, and if incubation be continued liquefaction of the medium usually occurs.

(*f*) *B. Hofmanni* in 18 hours: Yellowish growth well marked. The medium becomes markedly yellow and on continued incubation there is a general yellowish diffusion of tint (= alkalinity) throughout the medium.

(*g*) *Streptococci* derived from throat cultures: Very slight acid production, if any, and the pink tint rarely diffuses away from the colonies. When diffusion occurs the tint spreads in a patchy or streaky manner in the immediate neighbourhood of the several colonies. This medium seems to favour the free growth of *Streptococci* very markedly.

The 'A' medium has been used as a routine measure for the purpose of detection of the diphtheria bacillus in a very large number of throat cases, and, as a result of experience, it seems possible to say that where no bluish-red coloration appears after 18 hours' incubation of the inoculated medium *B. diphtheriae* is absent. Conversely it is possible, after a very short experience, to differentiate by means of the several tints produced the various organisms described, always taking into consideration the naked-eye appearances of the culture after the first 18 hours of incubation at blood heat. *B. diphtheriae* seems to be specially

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favoured by this medium, and next in order *B. Hofmanni* and *Streptococci* when present in pure culture.

Even in the case of admixtures of organisms there is shown on incubation a decided preferential action favouring first of all the diphtheria bacillus, and next in order *B. Hofmanni*, the *Staphylococci* and *Streptococci*. In pure culture *B. subtilis*, *B. megatherium* and *Torulae* flourish apparently unrestrained, but if they be present as a slight or accidental contamination of the pathogenic organisms cited—the differentiating and selective action of the medium in favour of such pathogenic varieties, continuing markedly during the first 18 hours of incubation, is sufficient to enable a ready naked-eye diagnosis to be made at the end of that period. On continued incubation the non-pathogenic varieties tend to overgrow and mask by their effects the presence of the pathogenic forms.

Where difficulty is experienced in distinguishing the several tints at close range it is often of considerable service to view the cultures at some little distance when the bluish-pink variety of tint peculiar to colonies of *B. diphtheriae* becomes specially noticeable.

*Calcium Chloride* added to the medium ('A') has the effect of increasing the intensity of the tints produced, and, in addition, the characteristics of the several growths cited appear to be more marked. The presence 1 % of the salt seems sufficient for the purpose. If, however, 2 % of the salt be added there is a marked restraint of growth of *B. Hofmanni*. In the case of the various *Staphylococci* production of the natural pigment in the raised colonies seems to be unusually well marked. (This latter phenomenon may have some relationship to the fact that the local water used for general laboratory work is excessively soft and deficient in lime salts, and pigment production by the various *Staphylococci* on ordinary agar media is usually not well marked.) If $CaCl_2$ be present in quantities exceeding $2\frac{1}{2}$ % there is marked restraint of growth of *Torulae* and *B. diphtheriae*.

Of the groups of four organisms cited, *Staphylococci* are least restrained by the presence of $CaCl_2$, but are altogether inhibited when the quantity present exceeds 3 %.

Calcium chloride causes the medium to assume a firmer and whiter appearance on coagulation and the medium seems to 'keep' better for longer periods. The apparent intensification of the several tints and the better marked characteristics generally of the various growths may be apparent rather than real and due to the increased whiteness of the medium.

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*Glycerine* added to the extent of 5 % to the 'A' medium appears to have the effect of delaying drying of the medium when prepared as slopes in tubes or in plates, and, moreover, adds to the nutritive and selective value of the medium so far as the diphtheria bacillus is concerned. Occasionally the intensity and rate of diffusion of the specific colours produced by the colonies of the various organisms described are better marked on glycerine 'A' medium than on the plain 'A' medium. Neither with the glycerine 'A' medium nor with the calcium-chloride 'A' medium have there been sufficiently extensive tests carried out with organisms derived directly from throat swabs to indicate with any degree of certainty that either medium is of greater utility than the simple 'A' variety. There is an indication for the use of calcium-chloride medium on occasions when it is necessary to exclude *B. Hofmanni* on incubation from a mixture of organisms in order to facilitate the diagnosis of other organisms present and more particularly to detect the presence of *B. diphtheriae.*

Latterly to the 'A' medium there has been added but ½ % of glucose, and on the whole this modification has proved of greater service in that the tint produced by colonies of *B. diphtheriae* is more readily distinguished from those produced by colonies of other organisms especially during the first 18 hours of incubation.

Throughout these experiments the presence of *B. diphtheriae* has been confirmed by microscopic examination. A wet method has been used as follows: Films are made on slides, fixed, dried by heat, and cooled and covered with a drop of ½ % watery-methylene-blue for a few seconds. A cover-slip is placed over the drop and blotting paper pressed heavily on. The organisms are then examined by means of an oil-immersion lens and their characteristic morphological appearances and arrangement noted. Thereupon while still under observation a drop of ½ % acetic acid is placed at the side of the cover-slip and differentiation shortly occurs, the beaded appearance of the organisms giving way to decolorisation with the appearance of dark granules—polar bodies—characteristic of the bacilli. I have occasionally been misled by the appearance of organisms resembling morphologically *B. diphtheriae* and indistinguishable therefrom, such organisms being a contamination of the watery-methylene-blue first added. Such organisms however are not fixed on the slide and on careful focussing they may be seen to be floating immediately below the lower surface of the cover-slip. In such cases it is always better to renew the watery-methylene-blue in use at short intervals and sterilise the bottle on each occasion.

The polar bodies of *B. diphtheriae* grown on glycerine 'A' media seem specially well marked, and the bacilli are more regular in shape, there being less tendency to the production of involution forms.

Certain strains of acid-producing *Staphylococci,* especially those obtained after repeated subculture on the ordinary laboratory media,

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| Medium, Blood serum + 25 % Broth + Glucose ½ % + Neutral red | Control, uninoculated colour | Incubation at 37° C. for 18 hours | | At 37° C. for 36 hours | |
|---|---|---|---|---|---|
| | | *B. diphtheriae* (No. 1633) | Marked and producing *Staphylococcus* (No. 1900) | *B. diphtheriae* (No. 1633) | *Staphylococcus* (No. 1900) |
| ½ % Boric acid = R ½ | Bright yellow | Pink growth; some little diffusion of tint into surrounding medium | As with *B. diph.* | Growth colour as at 18 hrs. but greater diffusion | As with *B. diph.* |
| 1 % Boric acid = R 1 | Faint pinkish yellow | Pink growth; some diffusion | Slight localised pink growth; no diffusion of tint | As at 18 hrs. but increased diffusion | As at 18 hrs., slight diffusion |
| 2 % Boric acid = R 2 | Faint pink tint | Pink growth; very slight diffusion | Localised pink growth; no diffusion | Pink growth; some diffusion | Pink growth; very slight diffusion |
| 1 % KCNS = A | Yellow ... | Pink growth; some bluish-pink diffusion | Pink growth; very little diffusion | Overgrown with *B. subtilis* | Pink growth; more diffusion |
| AR ½ ... | Yellow ... | Pink growth + diffusion | Pink growth; slight diffusion | Pink growth + bluish - pink diffusion | Pink growth + pink diffusion |
| AR 1 ... | Faint pinkish-yellow tint | Pink growth; slight diffusion | As with *B. diph.* | Increased growth and diffusion | As with *B. diph.* |
| AR 2 ... | Faint yellowish pink | Faint pinkish growth; free broth in tube acid | Yellow - pink growth; free broth alkaline | Increased pink tint | As at 18 hrs. |
| Potassium ferricyanide ½ % = I ½ | Yellow ... | Deep pink growth; general diffusion of tint | Yellowish pink growth; slight diffusion of tint | As before ... | As before, but increasing diffusion of tint |
| $K_3FeCy_6$ 1 % = I 1 | Faint red pink (cerise) | Pink growth + diffusion of tint | Whitish growth on pink base + some diffusion | As before ... | Tint and growth yellowish |
| $K_3FeCy_6$ 2 % = I 2 | Brown pink (medium soft) | Pink growth ... | Brownish pink growth | Colour approaching coccus growth | As before |
| AI ½ ... | Yellow ... | Deep pink growth and diffusion of tint | Yellow - pink growth + diffusion | As before ... | Yellowish tint disappearing but difference still marked |
| AI 1 ... | Brownish - yellow (medium soft) | Rich pink growth and bluish - pink diffusion | Yellow - pink growth on a dull cerise base | As before ... | As before, but growth becoming pinker; difference between cultures still very marked. |
| AI 2 ... | Greenish - yellow brown | Reddish growth + colour of medium | As colour of medium | Reddish tint fading | As before, very little difference between cultures |
| Potassium ferrocyanide 1 % = O | Bright yellow | Very faint pink growth; very slight diffusion of colour | Pink growth; slight pink diffusion | As before | As before |
| AO ... | Yellow ... | Pink growth and some pink diffusion | Slight pink growth & slight localised diffusion | Increased growth and diffusion | Increasing depth of tint and diffusion |

The value of the media in differentiating growths by means of colour reaction (including diffusion of tint through media) is in the following order:—A, AI ½, I ½, AI; RI, AR ½, AO; I 1, R 2; the first three being specially valuable during the first 18 hrs. of growth, and the two last showing up differences after 36 hrs. incubation.

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occasion some difficulty in naked-eye diagnosis when incubated on the modified 'A' medium, and the tints produced approximate very closely to those furnished by the colonies of *B. diphtheriae* at the 18th hour of incubation. Further incubation up to the 24th hour is, as a rule, sufficient to overcome this difficulty.

The following experiments show in greater detail some methods which serve to differentiate the appearances of colonies of acid-producing *Staphylococci* from those of *B. diphtheriae* on 'A' and other media.

It is somewhat remarkable that potassium ferricyanide when present to the extent of 1 % in blood serum does not inhibit the growth of *B. diphtheriae* or of certain *Staphylococci*, whereas when the quantity exceeds $\frac{1}{8}$ % in nutrient agar complete restraint of growth occurs.

To complete the series Boric acid (= R) from $\frac{1}{2}$ % to 2 % has been added to neutralised-glucose-bouillon-serum both with and without sulphocyanide of potassium and various organisms sown with the following results. (Period of incubation, three days at 37° C.)

*B. Hofmanni*: on media R $\frac{1}{2}$, and AR $\frac{1}{2}$, fair growth, many discrete colonies; on media R 1 and AR 1 slight growth only; on media R 2 and AR 2, very slight growth indeed.

*Staphylococcus p. aureus*: pink growth with some pink diffusion on all the combinations tested.

*B. megatherium*: some growth on all the combinations tested; many spores present; some pink diffusion.

*Torulae*: very slight growth indeed on media containing R $\frac{1}{2}$ or R 1.

I wish to express my deep indebtedness to Dr T. Thomas Rankin, Senior Medical Officer of the Leeds City Fever Hospitals, for the trouble and care he has taken in applying independently some of the foregoing media to the diagnosis of *B. diphtheriae* and associated organisms in cases under his care.

Up to date over 6000 tests have been made, 3000 of which have been with the modified 'A' medium alone. With this modification there has not been a single instance in which it was not possible to diagnose the presence or absence of the diphtheria bacillus without the aid of a microscope, and solely by means of the naked eye. In particular, it is possible to state that when the specific coloration is absent after 18 hours' incubation at blood heat, the absence of *B. diphtheriae* may safely be assumed.

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To dispense with the microscope in the routine examination of cases of suspected diphtheria—either for the detection of carrier cases or in the examination of swabs from likely cases of diphtheria among the general population—would mean a saving of at least 90 %; while in the routine examination of cases of diphtheria already isolated, either for the purpose of confirmation of diagnosis or with a view to the discharge of patients apparently convalescent of the disease, there would be a saving at least of 50 % of the time and labour now involved.

For the latter purpose as well as for the routine detection of cases of post-scarlatinal diphtheria in scarlatinal isolation wards the modified 'A' medium seems specially suitable.

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