OBSERVATIONS ON THE GROWTH OF MENINGOCOCCI IN VITRO IN RELATION TO VIRULENCE.¹

A REPORT TO THE MEDICAL RESEARCH COUNCIL ON WORK CARRIED OUT AT THE UNIVERSITY OF CAMBRIDGE PATHOLOGICAL LABORATORY AND FIELD LABORATORIES.

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(h) The Accessory Growth Factors.

It is well known that in primary culture the meningococcus requires these accessory substances and probably this is the principal reason why it is universally understood that special media are necessary to grow it. The absolute need for special provision of these accessory factors decreases with the time a given strain has been kept in culture and the meningococcus can readily be educated to grow on “ordinary media,” as is a common practice in laboratories. But two outstanding features of the life of this organism in vitro definitely prevents any application of this practice to the work we are doing at present: firstly, freshly isolated strains or those which have been raised in virulence will not grow readily on ordinary media; and, secondly, the repeated subculture necessary to accustom the organism to such media immediately deprives the cultures of their power to kill mice. Thus it is essential, apart from any other properties the medium may possess, to provide the required accessory growth factors.

It is not our purpose to consider these substances with any intention to classify them, but the manner in which we used the fluids containing them falls naturally into two categories:—

1) Their addition to the medium immediately before use and after sterilization.

2) Their addition as an integral part of the preparation of the medium before sterilization.

The first method requires that the substances be added to the otherwise finished medium with strict sterile precautions and at the same time exercising minute supervision over other conditions, in order that the supposedly delicate accessory growth factors are not subjected to treatment which either destroys or removes them: such as autoclaving or adsorption by large surfaces provided by filter paper or finely divided precipitates. The second method allows of the accessory growth substances, in the presence of the other constituents of the medium, to be subjected to the usual processes of sterilization, filtration, etc., without interfering with their function. Obviously this has great advantages.
The substances we have tried by the first method are: ascites fluid, horse serum, laked horse blood, formol serum and extracts of red corpuscles. The substances we have tried by the second method are: Gordon's extract of pea-flour, freshly drawn horse blood, extracts of red corpuscles, formol serum and our own method of using extracts of heart muscle.

So much for the categoric treatment of the subject, and we shall now consider each of the individual substances by their effect upon growth.

Ascites fluid, when freshly drawn and added immediately before use to agar media which have been cooled to \( 45^\circ \) C., is undoubtedly superior to any other substance we have tried. In our experience it enables media which are otherwise absolutely unsuitable for meningococcal growth to give a remarkable yield per unit area. Nevertheless, in addition to the difficulty of maintaining an adequate supply and contrary to the invariable statement of the case, ascites fluid does not keep well exposed to the air in vessels plugged with wool. We have not tried hermetically sealed vessels. Using the same batch of medium, or batches made as nearly alike as we know how, to which was added 5 per cent of ascites fluid immediately before pouring the plates, we obtained profuse, smooth, creamy growth when the fluid was freshly drawn. As the fluid aged (ten days) the growth tended to become sticky, until at last it became unmanageable, reaching such a degree of stickiness that it could not be removed from the surface of the agar (thirty days). As the sliminess of the growth increased the yield appeared to become less. We were inclined, at first, to blame every process and ingredient used in making the medium except the ascites fluid, and we spent a considerable time investigating the effect of various alterations, without improving matters until we examined the ascites fluid. Ascites fluid which had been kept in vaccine bottles with wool plugs for thirty days was very alkaline, well beyond \( pH 8.0 \); when it was fifty days old, five cubic centimetres of it required 0.7 cubic centimetre \( N/10 \) HCl to adjust its reaction to \( pH 7.2 \), in spite of the fact that the fluid appears to be comparatively slightly buffered. When the reaction of five cubic centimetres of this fluid containing phenol red was adjusted to \( pH 7.2 \) and left standing in a boro-silicate glass tube for fifteen hours exposed to the air, the upper layer of the fluid became very much more alkaline than the lower layer.

A hundred cubic centimetres of ascites fluid were placed in a wool-plugged flask and the reaction was adjusted to \( pH 7.2 \) with full sterile precautions; it was then left in a cool dark cupboard for six days, when the reaction was found to be \( pH 8.0 \) and five cubic centimetres required 0.3 cubic centimetre \( N/10 \) HCl to restore the reaction to \( pH 7.2 \). Every precaution was taken to reduce the absorption of alkali from the glass. Two litres of the same fluid were stored in the cupboard in a Winchester quart bottle with a rubber bung and only exposed to the air through a piece of glass tubing of four millimetres diameter tightly plugged with wool; after fifty days it was more acid than \( pH 6.6 \) and 5 cubic centimetres
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required 0.2 cubic centimetre N/10 NaOH to adjust its reaction to pH 7.2.

The growth obtained on good media when 5 per cent. of the fluid which had become alkaline was added, was too sticky to remove from the surface of the agar; neutralization of the fluid (to pH 7.2) before using it gave smooth creamy growth once more.

It appears, therefore, that exposure to the air quite rapidly causes a change in ascites fluid, which renders it more than ordinarily difficult to use and for that reason we abandoned it.

Horse Serum and Laked Horse Blood.—The serum was obtained in the ordinary way by allowing sterile blood to clot. The laked blood was obtained by drawing horse blood into an equal volume of sterile distilled water; it haemolyzed immediately, subsequently a clot separated and the haemoglobin-stained serum was used to supply the accessory growth factors. Both these substances were added to the medium immediately before use in quantities varying between one and five per cent and they stimulated growth nearly, but not quite, as well as human ascites fluid.

We discontinued their use because they exhibited a similar change to that described in ascites fluid and with the same effect, without presenting any other property of particular advantage.

Formol Serum was prepared as described by Nicolle, Debains and Jouan (1918), Legroux (1920), used horse serum. It was added to our media immediately before use in the quantity directed and our results were less satisfactory than those obtained with ordinary serum or laked blood. This substance also became alkaline when exposed to air and again after neutralizing.

It is a curious fact that formol serum prepared as directed does not clot when autoclaved, it becomes more opalescent and a smell of H₂S is evolved; and that whole serum to which two per cent of formalin has been added does not coagulate when heated at 100°C, or even when boiled, but when added to medium containing two per cent of agar and our usual salts coagulation takes place on steaming or autoclaving.

Extracts of Red Corpuscles, prepared in the manner described by Agulhon and Legroux (1918), Legroux and Mesnard (1920), when added to media immediately before use gave results slightly inferior to those yielded by our ordinary media, to which the accessory growth factors were added before sterilization. The only remark we have to add is that the addition of the extract before autoclaving only slightly reduced its efficiency. These extracts were designed to replace fresh blood in media on which it was required to grow Pfeiffer’s bacillus.

Gordon’s Extract of Pea-flour was used in preparing “Trypagar” as described by Gordon, Hine and Flack (1916). We did attempt a few experiments in which this extract was added to our media, but with little success as the results were always inferior to our standard method.

When this substance was used by Gordon and others, when the medium
was supplied on a large scale during the epidemic of cerebro-spinal fever in the British Army, it was always accompanied by horse serum or laked rabbit blood, to be added to the medium before use. This fact made us less interested in it than we would have been had it unfailingly grown the meningococcus in primary culture without the additional substances.

_Freshly drawn Horse Blood._—The addition of fresh blood to media is a well-established method of promoting the growth of delicate organisms and it is usual either to add it to the melted and cooled medium, or to smear the surface with blood, but each method suffers from inconveniences we wish to avoid. The coagulation of blood in the medium by heat has had its advocates. While certain of them are content with an opaque medium rather resembling a slab of chocolate, others have removed the coagulated protein by specialized methods with the idea of retaining the accessory growth factors, which otherwise, they claim, are destroyed or removed (Lloyd, 1916). The method we advocate, which has given results only surpassed by _freshly drawn_ ascites fluid, and perhaps the method we shall presently describe, depends upon the coagulation of fresh blood in the presence of agar and the subsequent removal of the clot, without interfering with the accessory growth factors required by the meningococcus in any degree we can detect. At the same time the method has this advantage, that no elaborate nor troublesome technique is required either for the removal of the coagulum or the sterilization of the finished medium. When all the ingredients of the medium have been added and the melted agar has been cooled to 50°—55° C., seven per cent of freshly-drawn horse blood is added, with stirring to make a homogeneous mixture, which is then gradually raised to 100° C. in the steamer. By keeping it at this temperature for thirty minutes a considerable shrinkage of the coagulum is produced. The agar is then allowed to cool and set and so is _left overnight_. We consider it to be important to let the agar set in the presence of the coagulated blood, as we have tried on many occasions the effect of removing the coagulum immediately after heating at 100° C. and without preliminary setting of the agar, but the result has always been most unsatisfactory. It is possible that the alteration of the physical state of the agar during setting plays some part in the process. The next morning the agar is melted, decanted off from the coagulum which will have contracted further and strained through surgical lint in a hot-water funnel; finally the clot is tipped into the filter and after draining the remaining medium is gently wrung out by twisting up the lint. After the reaction of the filtrate has been adjusted to pH 7.2, it is distributed as required and sterilized in the autoclave at 120° C. for twenty minutes. The resulting medium is glass clear, although there may be a small flocculent deposit which rapidly settles out, and the virulent meningococcus grows readily in primary culture. The removal of the coagulum is facilitated by carrying out the process in straight-sided enamelled pails, within which are placed closely fitting muslin bags, held in position by strips of cane. When coagulation has taken place the
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strips of cane are removed and the bag is gently drawn to one side; the bag holds back the clot while the medium is decanted, and eventually, after draining, the bag and contained clot are wrung out into the lint filter.

We wish to comment on a few points arising out of this method:—

The quantity of blood used is the maximum which we found gave us a manageable coagulum; greater quantities were inconvenient and did not seem to possess any advantage. Better results seem to have been obtained when the reaction of the medium in which the blood was coagulated had been adjusted to pH 7·0—7·2, rather than when it was definitely acid or alkaline.

We tried various methods of separating the coagulum, such as filtration through glass wool, paper and lint, also simple decantation, but we were unable to detect any difference in the growing power of the product. Filtration through lint possesses the great advantage of being a rapid process with very little loss of material.

Sterilization by steaming at 100° C. on three successive days showed no advantage over autoclaving. In fact we find that this medium stands several autoclavings on different occasions at 120° C. for twenty minutes, without appearing to change in any way other than becoming clearer.

In the course of our work it has frequently been necessary to make a series of media, in small amounts, in which some one constituent varied. This was greatly simplified by the fact that the coagulation of blood in a solution of agar in extract, with the subsequent addition of the other constituents, was quite as effective as coagulating the blood in the otherwise finished medium.

We call this form of medium “EE agar” (Extract, Blood) and “EDB agar” when the digest has been added.

Fresh Heart Muscle.—Under certain conditions it is difficult to get a sufficient quantity of freshly drawn blood to make large amounts of medium in the manner described, and it occurred to us to try to use the heart muscle from which we make our extract and digest. But before proceeding we wish to repeat that we only use freshly killed meat.

Attention has already been drawn to the copious coagulum we observed in making our ordinary extract, when it was raised to 100° C., but we wish now to emphasize another closely related point. When the meat has been extracted at 70° to 75° C. for three hours the fluid is a rich tawny red colour, which changes when heated at 100° C. to a pinkish yellow, with the separation of the coagulum and on autoclaving a further change of colour to a bright yellow takes place, with the separation of a fine whitish precipitate. At each stage the fluid has a bright, crystal-clear appearance.

We therefore filter the extract immediately after its three hours at 70° to 75° C., in the manner described under the method of making the extract, and into this filtrate we stir an equal volume of a 4 per cent solution of agar in ordinary extract cooled to 70° C. The whole is then raised to 100° C. and treated strictly in the manner described for coagulating blood in the
medium, with the exception that the muslin bags are dispensed with because there is not so much coagulum to remove. The resulting filtrate is not so clear as when blood has been used but its transparency can be much improved by entangling the fine coagulum in a precipitate of phosphates, as described under methods of clearing medium. But we find it advisable not to clear the medium thoroughly as definitely better results are obtained when it is slightly cloudy.

This preparation, which we call "EH agar," forms the basis of our medium and it keeps well stored in this form after autoclaving. To it we add the desired quantities of digests and salts and adjust the reaction, to make the finished medium which we speak of as "EHD agar" (Extract, Heart, Digest). This medium is no whit inferior to "EDB agar," in fact we are rather inclined to judge it as better. The accessory growth factors in this form are resistant to autoclaving and we strongly recommend the method for making media for general purposes.

It is best to avoid autoclaving "EH agar" more frequently than is absolutely necessary as the finely suspended matter tends to agglomerate and settle out as a reddish deposit, leaving the agar perfectly clear and the growth obtained on this very transparent medium is erratic and in the case of highly virulent strains it may be uncertain. In this respect "EHD agar" differs from "EDB agar," which is unaffected by thorough filtration or the clearing effect of repeated autoclaving.

If, however, the deposit is added to part of a plate of perfectly clear "EHD agar," satisfactory growth occurs on that part of the surface overlying the deposit; and if the fine-precipitate is evenly distributed through the medium before use, perfectly satisfactory growth is obtained over the whole surface. For this reason we are content at present with the slightly cloudy medium obtained by filtration through lint and do not clear it by means of the phosphate precipitate or any other method.

(To be continued.)
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