THE GROWTH OF STREPTOCOCCI IN A FLOWING MEDIUM WITH SPECIAL REFERENCE TO HAEMOLYSIN PRODUCTION.

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(With Plate I and 2 Text-figures.)

The culture of organisms in a stagnant liquid medium, which is the normal method employed when the products of their metabolism are studied, has obvious disadvantages. The medium is constantly varying in its constituents and physical characteristics; prominent variations are oxygen tension, *p*H, food supplies and surface tension, and growth phase of the organism. Experimenters are constantly occupied in preparing nutritive media bearing closer analogy to living tissue fluids; but strangely enough little attention has been directed to maintaining the dynamic conditions obtaining when infection takes place. Yet such conditions may greatly modify the metabolism and general pathological activity of the organism.

Attempts to imitate *in vivo* conditions have often taken the form of the insertion of collodion capsules into the peritoneum of animals; these are later removed and their contents studied. Dialysis through collodion is, however, slow; and the technique employed has involved autoclaving the capsule, with very marked effect on the permeability of the wall.

Weiner (1927) described an elaborate method for automatic sub-culture, which was in some ways comparable to that here described. Haddon (1928) has described a continuous irrigation method of great simplicity and has used it in the culture of *B. pestis*. He suggests that irrigation allows organisms to proliferate constantly at the logarithmic phase. Ridley (1928) described a mixing device by means of which he found four times the number of organisms to be cultured in a given volume of fluid when compared with a control.

In this connection it is of interest to note that Winslow (1928) states that rivers contain 1000 organisms per c.c., while stagnant waters, such as lakes, contain on an average 50 to 150.

I find no reference to the study of metabolic products, such as toxins or haemolysins, by these means apart from the collodion capsule method.

I have used a number of devices to maintain flow of medium during the growth of an organism. These have been:

(1) The growth of organisms in collodion sacs of great permeability (regulated by Brown's method (1915)), immersed in a large bulk of medium.

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(2) Growth in a vessel divided into two compartments, separated by a collodion plate; flow of medium was obtained by suction, and fresh medium added from a drip feed.

(3) Growth in a series of bulbs, joined together, flow of medium being obtained as in (2); and finally:

(4) A combination of methods (2) and (3), by joining the series of bulbs to a vessel with two compartments, and using either a Seitz filter disc or collodion disc as filter.

Some results of interest were obtained by methods (3) and (4), and the technique used will be described.

The reservoir of medium is of 3-litre capacity. It is arranged as a Marriott's bottle, so that a constant head of fluid is assured. Arrangements are made for bubbling oxygen or other gases through the medium; and, if desired, a two or three way junction may be provided, so that a single culture may be irrigated with different fluids at will.

A screw clamp on a rubber tube connection (thick-walled pressure tubing) regulates flow.

Several types of culture chamber (*A*) have been used. Two are illustrated; in one, an upright conical vessel, medium is added from below and removed from above, or *vice versa*, as required; in the other, a series of bulbs, with a drip feed, are arranged with sampling tubes.

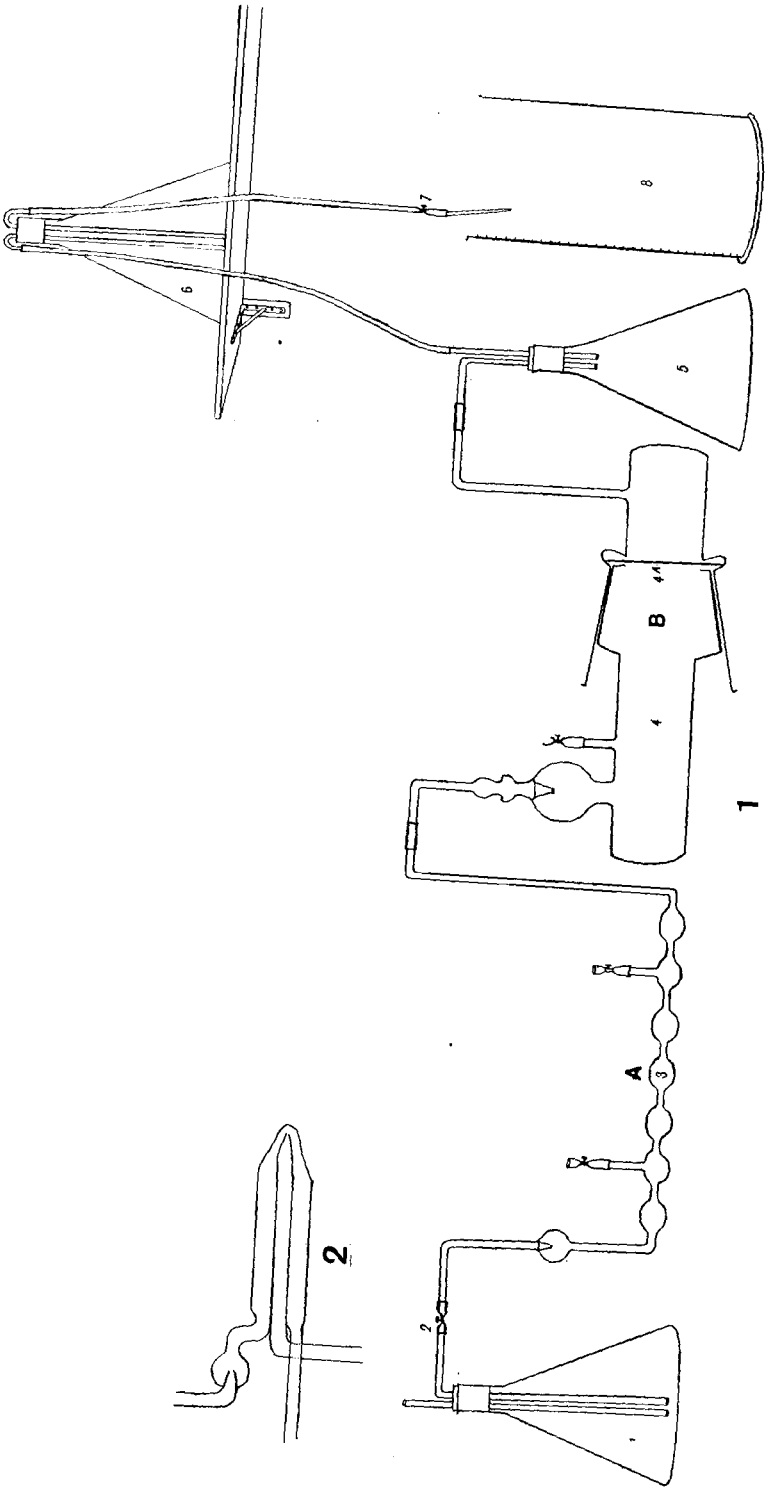
The second vessel (*B*), divided into two parts, may best be understood by reference to the illustration. The elaborate nature of the chamber is necessary to ensure a joint. A ground glass hollow piston fits into a ground glass cylinder, with a shoulder to fix the filters used; two holes above the filter are for the insertion of collodion dissolved in alcohol as a seal. When assembled, this apparatus is fixed in a frame. Silk or linen discs are used as backing for the filters.

Before use, all the components, with pressure tube connections, are wrapped and autoclaved. The apparatus is assembled over steam; the filter chambers are assembled, sealed with collodion, and filled with medium over steam. After 24 hours' incubation as a test of sterility, the organism is inoculated into chamber (*A*), and after 24 hours' stagnation, irrigation is begun by attachment to suction from a head of water. Flow is further controlled by a screw clamp and measured by collecting waste water in a graduated cylinder. Chambers (*A*) and (*B*) are in a water bath.

Many variations are possible; in particular a flask containing absolute alcohol may be substituted for apparatus (*B*), and thus any substances precipitable by this reagent may be so precipitated as formed, before they have had time to break down.

By incorporating an indicator in the medium, irrigation may be carried out at a rate producing any desired *p*H in the culture.

The most difficult problem is the control of rate of irrigation, and an improved method would be of value.



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Only three results will here be studied. They are:

(1) The nature and location of growth.
(2) The number of organisms grown.
(3) The production of haemolysin by streptococci.

The medium has always been 2 per cent. Bacto Peptone beef broth, *p*H 7·4. The organisms have been *Streptococcus scarlatinae* (Dochez), *Streptococcus pyogenes* (blood culture, laboratory strain). In both cases results were identical.

(1) *Nature and location of growth, using horizontal bulb culture chamber.*

After 24 hours, sedimentation and auto-agglutination take place in a stagnant culture. Irrigation is then begun. Growth does not take place uniformly in every bulb. Whatever the rate of irrigation and however long (up to 10 days) it is continued, it is most marked in the first bulb, and then, by even gradation, drops until in the last bulb it is very slight. Since rate of flow does not modify the location of growth, sedimentation does not produce this result.

(2) *The number of organisms grown.*

Vast numbers of organisms may be grown in this chamber. After 10 days it contained 6000 millions per c.c., as compared with 300 millions in a control flask of 100 c.c., grown stagnant for the same time.

But this increase is only apparent; few organisms grow in chamber (*B*). If the overflow fluid is collected in a simple flask at 37° C., it is found that any unit volume of fluid grows, in ten days, twice as many organisms as a control stagnant culture.

The organisms in this chamber stain strongly by Gram's method after ten days' irrigation, and involution forms are scarce. On sub-culture on to solid media the remarkably rich growth indicates that the majority are alive.

It might be supposed that such a rich culture, in the case of a toxin producing streptococcus, might, if irrigation were stopped, and stagnation for varying periods substituted, give evidence of greatly increased toxigenicity. This is not so: neither on filtering the contents of the culture chamber immediately after irrigation ceases, nor by any subsequent stagnation for periods from one hour to three days has any toxin capable of killing a rabbit in doses of 2·5 c.c. been obtained.

On the other hand, the filter chamber, when a Seitz filter disc was used to separate the compartments, was found to contain toxin capable of killing a rabbit in a dose of 2·5 c.c. administered intravenously; irrigation had proceeded for 72 hours when samples were taken, and filtered. Haemolysin was absent from the filtrate. Therefore, though growth was very vigorous in the culture chamber, toxin was not demonstrated, either when the medium was sampled while flowing or after varying periods of subsequent stagnation;

growth was poor in the filter chamber, but toxin was there demonstrated and at high titre.

The object of the filter chamber was to imitate, in some sense, the condition which might obtain when a streptococcus grows in an abscess cavity; similarly, the culture chamber was analogous with a blood stream infection.

The collodion filter disc was intended to concentrate the colloidal toxin; but it is very difficult to obtain a steady brisk flow of medium, as high pressures are required. A Seitz filter, of course, merely dams up the organisms in the chamber; it cannot concentrate the toxin.

Although the whole apparatus was designed for the study of toxin formation and its conditions precedent, further studies on this point have not been prosecuted. It was found possible to concentrate the toxin with absolute alcohol, and in this simple way to obtain what was sought—a reliable rabbit-killing toxin (Pulvertaft, 1928).

The following experiments were, however, performed:

(1) It was considered possible that a very labile toxin, hitherto not identified, might be formed and rapidly broken down. This breakdown might be prevented by collecting the fluid, as it came over from chamber (*A*), in a flask over ice.

Irrigation was therefore carried out at a rate giving a turnover of fluid in chamber (*A*) once in 12 hours. After three days the fluid in the flask, kept on ice, was filtered, and injected into pairs of rabbits, in doses of 10 c.c., 5 c.c. and 2·5 c.c. intravenously. No symptoms followed.

If, however, the flask is kept at 37° C., instead of 0° C., doses of 5 c.c. kill rabbits on intravenous injection; such deaths were invariably prevented by scarlet fever anti-toxic serum.

(2) It was considered possible that such labile toxin might disappear, even at ice temperature. This disappearance might be prevented by instantaneous precipitation by ice-cold absolute alcohol or saturated ammonium sulphate.

Irrigation was carried out at a rate giving a turnover in chamber (*A*) once in 12 hours. The fluid was collected as it came over in absolute alcohol, over ice, and in saturated ammonium sulphate.

Irrigation was continued for seven days. The alcoholic precipitate was re-dissolved in saline at 37° C., in a volume equal to one-tenth the volume of medium used in irrigation and injected intravenously into rabbits. 2 c.c. was found to be a fatal dose; death was prevented by anti-toxin.

The ammonium sulphate precipitate was dialysed, with 0·25 per cent. phenol, against distilled water, and made iso-tonic with red cells by means of NaCl, but was non-toxic in any dose up to 10 c.c. Dialysis took one week and the use of phenol was ill advised owing to its deleterious action on toxins.

Thus there is no evidence that a very labile toxin exists; and wherever toxin was demonstrated, scarlet fever anti-toxic sera completely inhibited its action.

It is worth while noting that, though the fluid, precipitated as it came over from chamber (*A*), contained toxin, it produced no symptoms in rabbits other than those produced by toxin precipitated by absolute alcohol, and it was neutralised by scarlet fever anti-toxic serum. Thus there was no evidence that any substance of toxic properties was made, but rapidly broken down; this assumes that such hypothetical substances are precipitable by alcohol. Moreover, such substances were not demonstrated by collecting the overflow fluid on ice without precipitating it.

(3) *Haemolysin experiments.*

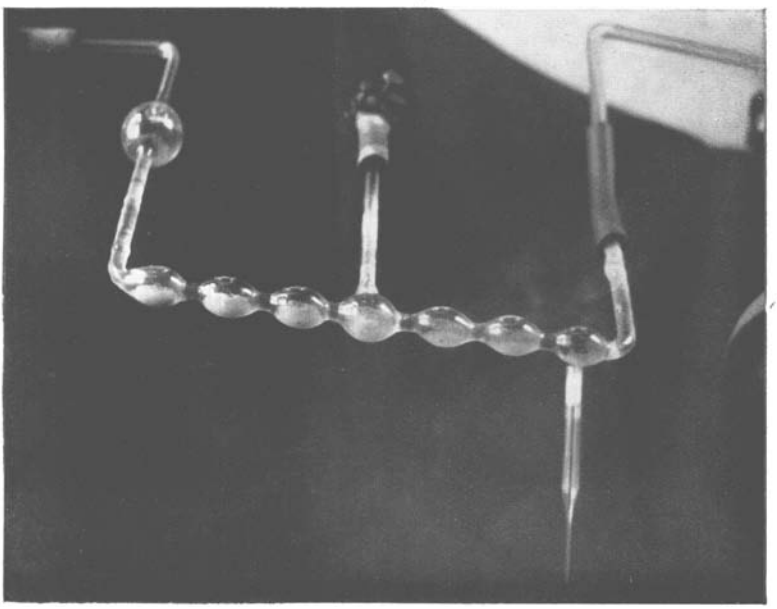
The most interesting finding was that the production of haemolysin is dependent on the rate of flow of the medium; keeping it constant at a rate giving a turnover of the culture chamber fluid once in 12–24 hours, haemolysin gradually disappears. If now the rate is raised so that the turnover is once in 6 hours, haemolysin appears again and at this rate of irrigation remains present at full titre for 30 hours. Whether it appears and disappears at any rise in the rate of flow, or whether it remains constant when a certain optimum rate is used, is not yet determined, but the evidence is in favour of the latter view.

TECHNIQUE.

2 c.c. samples of culture were taken with a Pasteur pipette and spun down for 10 minutes in a centrifuge. The supernatant fluid was then put up against 0·5 per cent. sheep cells at 37° C. for 1 hour, in dilutions of 1 in 2, 4, 8, 20, 40 and 80. The highest titre found, when serum was not used in the medium, was 1 in 20; when serum was used, lysis was found up to 1 in 160.

In their paper on streptococcal haemolysin, Cesari, Cotoni and Lavalle (1927) give many graphs of haemolysin production, and its appearance at or about the sixth hour of stagnant cultures, with a later drop to zero, has been many times confirmed by me. I find, however, that its appearance can be prevented by growth at unfavourable temperatures or in unfavourable media. These several findings suggest that haemolysin production is related to a certain growth phase of the organism, and this question is now being investigated. This has, in fact, been suggested by Neill and Mallory (1926), who found that its presence in cultures could be prolonged by culture in the presence of yeast extracts. Meanwhile, by this continuous flow method here described it is evident that one great modification of conditions obtaining in stagnant cultures can be induced; haemolysin can be produced or inhibited at will. It may well happen that in human infections haemolysin may similarly be produced or fail to appear as conditions vary: *e.g.* when a septicaemia leads to abscess formation, or *vice versa.*

In conclusion, it may be stated that the technique here given is simple and contaminations few, and that it is a ready method of continuous variation of cultural conditions in a great many different ways, while keeping the organism constant.



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DISCUSSION.

The fact that haemolysin is produced in early stagnant cultures and falls in later ones to zero, suggests that its production is related to certain growth phases.

The fact that rate of flow in the apparatus controls haemolysin production suggests that rate of flow controls the growth phase.

I find that toxin is not found in early (6–24-hour) cultures but is present in 3-day cultures. Using, as I invariably have, a medium without serum I find the presence of haemolysin almost always to indicate absence of toxin at that stage of growth.

Thus toxin production is probably related to another growth phase.

As a hypothesis, I suggest that haemolysin is related to the logarithmic phase, and toxin to the negative acceleration or maximum stationary phase.

Thus, if rapid flow favours haemolysin production, a very much slower flow should favour toxin production. This probably accounts for the high titre toxin in the filter chamber, when the turnover of fluid is relatively slow. The hypothesis has been confirmed by using a very slow rate of flow in the culture chamber and collecting the overflow in absolute alcohol; the re-dissolved precipitate contained high titre toxin.

My thanks are due to Sir Cuthbert Wallace and Prof. MacLean for facilities in carrying out this research. The glass apparatus was made by Messrs Flaig and Sons and my assistant, Mr P. Carter, to whom my thanks are also due.

LEGENDS TO FIGURES.

Fig. 1. (1) A 3-litre flask, fitted with two tubes, both going to the bottom of the flask. The open one is plugged with cotton wool. This is the reservoir.

(2) A screw clamp, operating on pressure-tubing, to control flow of medium.

(3) The culture chamber, fitted with sampling tubes and drip feed.

(4) Filter chamber, fitted with drip feed, sampling tube and filter (4 *A*). Two holes (not shown in figure) allow collodion to be poured in to seal filter.

(5) Overflow vessel.

(6) Pressure head of water; flow controlled by clamp at (7) and measured in cylinder at (8). On releasing clamps at (2) and (7), medium flows at required rate from (1) to (5).

Fig. 2. Another type of culture chamber, with drip feed; the exit tube leads to the bottom of chamber, thus removing sedimental organisms.

DESCRIPTION OF PLATE I.

Culture chamber after 14 days' continuous irrigation, showing gradation of growth.

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