A Practical Liquid Medium for Cultivation of *Trypanosoma cruzi* in Large Volumes

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A PRACTICAL LIQUID MEDIUM FOR CULTIVATION OF TRYPANOSOMA CRUZI IN LARGE VOLUMES

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In the media usually employed for the cultivation of *Trypanosoma cruzi*, the portion most favorable to the growth of this microorganism is the water of condensation formed on blood agar. This fluid is so small in amount that rubber caps are placed on tubes and flasks to conserve it during the incubation of cultures. In 1936 Kelser used such a medium for the production of diagnostic antigen for Chagas disease and demonstrated that the antigen had merit as a medical tool. However, the necessity of having aseptically drawn blood makes the preparation of large amounts of Kelser's medium difficult.

In 1943 Miss Nylah Tom showed that a blood agar medium could be inspissated and sterilized in the autoclave at 15 pounds for 20 minutes. This discovery suggested the sequence of experiments by which we were able to arrive at a practical liquid medium for cultivation of *Trypanosoma cruzi* in large volumes.

In this laboratory, *Trypanosoma cruzi* has been cultivated in 5-ml, 500-ml, and 5-liter volumes in a solution of 2 per cent of peptone, 0.5 per cent of sodium chloride, and 0.2 per cent of glucose (pH 7.5). Previously coagulated and dried granules of rabbit red blood cells, placed on the bottom of the glass containers, supplied the additional nutrients required. Amounts of 50 to 100 mg of red cell solids per 5 ml of solution, 5 g per 500 ml, and 50 per 5 liters were found optimal. The granules did not appear to dissolve but remained on the bottom of the containers during growth. From 500,000 to 1,000,000 trypanosomes per ml were obtained.

Preparation of Granulated Coagulum of Red Cells

Pour 100-ml volumes of fresh rabbit red blood cells into 1-liter volumes of rapidly boiling distilled water, mix thoroughly, and maintain at the boiling point for about 10 minutes for complete coagulation. Collect the wet coagulum on cheesecloth filters, while hot, and discard the yellow filtrate. Transfer the coagulum, when semidry, to trays lined with adsorbent paper and continue the drying process for 48 hours in a 37 C incubator. Stir the coagulum at intervals to aid the formation of small granules. When thoroughly dry, the granules may be sifted for uniform size, ground finer with mortar and pestle, and stored indefinitely in a dry place. The granules used in our experiments contained 14.48 mg of total nitrogen and 0.28 mg of iron per 100 mg of solids (90.5 per cent of protein and 7.8 per cent of haematin). They were completely soluble in normal sodium hydroxide.

Preparation and Use of the Complete Medium

Medium used for the maintenance of cultures. Place 20 g of bacto peptone, 5 g of sodium chloride, cp, and 2 g of glucose, cp, in a 2-liter Erlenmeyer flask. Add 1 liter of distilled water and dissolve without heating. Add 3 ml of normal sodium hydroxide. The reaction should be pH 7.5.

Assemble 200 plugged and sterile 6 x $\frac{5}{8}$-inch tubes in test tube blocks. In each tube place approximately 100 mg of the dry, granulated coagulum of red cells, measured on the tip of a "Fischer scoopula"; 5 ml of the solution of peptone, glucose, and sodium chloride, and replace the plugs. Sterilize in the autoclave at 15 pounds for 20 minutes. This medium does not require the rubber caps usually employed to prevent evaporation of culture fluid during incubation. However, the tubes should be stored in the cold to prevent evaporation prior to use. The time required for the development of cultures depends upon the size of the inoculum, as shown in figure 1. Less than 10 organisms will give visible evidence of growth after an incubation period of 30 days. For the maintenance of cultures, we have used a 5 to 10 per cent inoculum (0.2 to 0.5 ml per 5 ml) and have obtained maximum growth and visible turbidity in 5 to 8 days, depending upon the age of the seed culture used.

Seed cultures were found to be satisfactory for two weeks following the development of visible turbidity. Older cultures remained viable as long as some turbidity remained in the tubes (at least two months) but lost their ability to initiate growth in a short period of time. Cultures were incubated at 28 C, and placed at room temperature after the development of visible turbidity.

Medium used for the assay of chemotherapeutic agents. Heat-stable chemotherapeutic agents may be diluted in a freshly prepared solution of peptone, glucose, and sodium chloride, and any desired number of tubes may be prepared as above for the testing of each concentration of chemotherapeutic agent.

Heat-labile chemotherapeutic agents may be diluted in a previously autoclaved solution of peptone, glucose, and sodium chloride, then sterilized by filtration and added to tubes in which the granulated coagulum has been sterilized in the dry form.

The seed cultures should be carefully separated from granular coagulum before use to prevent clogging of the pipette during the distribution of the inoculum.

Medium used for the preparation of Trypanosoma cruzi antigens. Place 5 grams of the granulated coagulum in the bottom of each of any desired number of 1-liter pyrex bottles. Add 500 ml of the solution of peptone, glucose, and sodium chloride to each bottle. Sterilize in the autoclave at 15 pounds for 30 minutes.

If the inoculum is 3 to 4 ml (approximately 1 per cent), the bottle culture may be expected to show visible turbidity in 10 to 12 days as shown in figure 1. The time required for growth may be shortened by using larger amounts of inoculum (25 to 50 ml) obtained from previous bottle cultures.

Five-liter volumes of medium. Place 50 grams of the granulated coagulum in

the bottom of 9-liter pyrex bottles. Add liters of solution and sterilize in the autoclave for 40 minutes. If the inoculum is 400 to 500 ml, the culture may be expected to show visible turbidity in 10 to 12 days.

EXPERIMENTAL DATA AND RESULTS

The granules of red cells have been titrated for activity by the use of varying proportions of dry material and of material redissolved in sodium hydroxide and sterilized by filtration. More than 2 per cent of dry material caused an un-

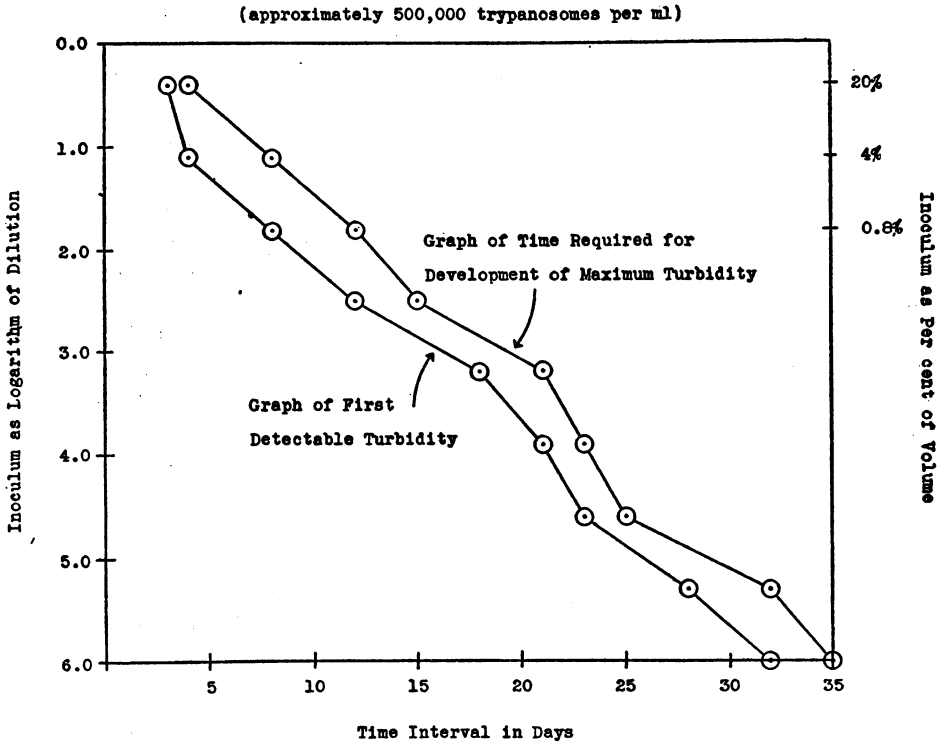

FIG. 1. THE EFFECT OF THE SIZE OF THE INOCULUM ON THE TIME REQUIRED FOR THE DEVELOPMENT OF VISIBLE TURBIDITY IN CULTURES OF TRYPANOSOMA CRUZI.

necessary loss in the volume of liquid medium which could be recovered in harvesting the cultures. As little as 20 mg per cent of redissolved red cell material could support growth, but the viability of such cultures was short. More than 100 mg per cent of redissolved red cell material caused inhibition of growth. Ability to support growth was restored when the material was caused to coagulate again by application of heat.

Several substitutes for red cell coagulum were found by placing the substances to be tested in dry form in the bottom of the tube and using solutions of peptone, glucose, and sodium chloride which contained 1 mg per cent of ferric protoporphyrin. The media were then sterilized in the autoclave and inoculated.

Refined preparations of thrombin, egg albumin, serum albumen, pseudoglob-

ulin, and globin were able to support growth. Fibrinogen, euglobulin, casein, and pepsin were inactive. Thrombin replaced red cell coagulum more nearly than did any of the other active proteins. Cultures developed in corresponding intervals of time and attained similar size. As little as 20 mg per cent of thrombin supported growth. In carrying out these tests, we could not depend upon the development of visible turbidity since varying degrees of turbidity occurred in the uninoculated medium, depending on the protein used. Ten tubes were prepared for each protein. The relative activity of the different proteins was judged by comparing the numbers of trypanosomes found in the culture fluids on successive days, using dark-field technique. The active proteins listed above are named in the order of preference. Of unrefined proteins, dried skim milk was able to stimulate early growth in fair numbers. Brewers' yeast extract was inactive.

Single amino acids such as *l*-valine, *dl*-leucine, *l*-isoleucine, and *l*-tyrosine were able to stimulate late growth and small numbers when used at 5 mg per cent. Only small numbers of trypanosomes developed in these media. Asparagine, *l*-cystine, and *l*-tryptophane were inactive in parallel tests. Titration of *l*-tyrosine showed an optimal range existing at 1 to 10 mg per cent. Larger concentrations were inhibitory.

Dr. R. W. Linton (personal communication) has pointed out that a medium ideally suited to *Trypanosoma cruzi* should permit the organism to grow as well at 37 C, the body temperature, as at 25 C. We have found that the complete liquid medium with granulated coagulum of red cells is able to support growth of *Trypanosoma cruzi* with development of visible turbidity through serial transfers at the higher temperature.

SUMMARY

A practical liquid medium for the cultivation of *Trypanosoma cruzi* in large volumes was obtained by placing a solution of peptone, glucose, and sodium chloride in contact with granulated coagulum of rabbit red blood cells. Other proteins, principally thrombin, were shown to have growth-promoting activity when used with ferric protoporphyrin. The organisms have been subcultured many times over a period of a year without showing any diminution in vitality.

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