NATURE OF THE CONTRIBUTION OF HUMAN SERUM TO THE GROWTH OF *GIARDIA* FROM THE RABBIT

MICHAEL DANCIGER

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APPROVED

[redacted]

ERNEST A. MEYER, Sc. D. (PROFESSOR IN CHARGE OF THESIS)

[redacted]

JOHN M. BROOKHART, Ph. D. (CHAIRMAN, GRADUATE COUNCIL)

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INTRODUCTION

A. Statement of Problem

Members of the protozoan genus Giardia are obligate parasites of birds, fish, reptiles, amphibia and mammals (1). The organism exists either in a dormant, resistant cyst form or in a motile trophozoite form. The pear-shaped trophozoite has four pairs of flagella, two morphologically similar nuclei and an adhesive disc with which it attaches to the epithelial wall of the small intestine (2,3,4), its domain in the host. The trophozoite can differentiate to the cyst form which is excreted with host feces. The life cycle is completed when excreted cysts are ingested by a new host in whose small intestine excystation takes place. Each cyst yields two trophozoites which proceed to divide by binary fission, and thus colonize the host intestine.

The incidence of this flagellate in man varies from 2 to 25% depending upon the locale (5); 7% of people in the U. S. were estimated to be parasitized in 1948 (6). Most people who harbor the organism express no clinical symptoms (2,6,7), although some minor intestinal damage may be occurring (4). Intestinal disease caused by Giardia is characterized by steatorrhea, weight loss and epigastric pain; malabsorption may also occur (3,8). Treatment with either atabrine or metronidazole usually results in prompt disappearance of symptoms and organisms (3,8).

In 1960 Karapetyan reported culturing Giardia from man for 7 months (9). The culture was initiated with chicken fibroblasts and Candida gulliermondii, but for continued growth of the flagellate only the Candida had to remain in the system. In 1962 the same worker reported

culturing Giardia from the rabbit for 5 months (10); in this case viable Saccharomyces cerevisiae was necessary. Meyer and Pope reported growing the parasite from the rabbit and chinchilla in mixed culture with S. cerevisiae (1), and Meyer has recently reported axenic culture of these two and a third Giardia from the cat (11). All three types of the protozoan are currently being grown in a medium composed of Hanks' balanced salt solution, the tissue culture medium NCTC-135 (see appendix), reducing agents, antibiotics, yeast extract and human serum.

The purpose of this work was to determine the contribution of human serum to the in vitro growth of Giardia originally isolated from the rabbit.

There were two main reasons for this endeavor:

1. A step toward a chemically defined growth medium.

   Large variations in growth and metabolism can occur in other cultured cells with only small changes in medium components (12,13, 14 for example). In the growth medium for Giardia small changes in the serum constituent from one pool to the next may well occur and may result in differences in the metabolic behavior of the organism. Further, the complete nutritional requirements of the protozoan can only be determined in defined medium.

2. Relation to the in vivo situation.

   Early experiments described in the Results section showed that the serum components responsible for Giardia growth were not dialyzable. This implicates macromolecules or substances closely associated with macromolecules. Since certain proteins found in

serum are also found in the small intestine (15) the possibility of the parasite utilizing them *in vivo* is real.

B. Survey of the Literature

1. Use of serum in culture media.

Serum is part of the growth supporting medium for many different kinds of *in vitro* life. Frequently, it is the only undefined constituent of growth medium for mammalian cell culture (16,17,18 for example). It is also part of the medium supporting the growth of several different pathogenic bacteria (19,20,21), and many of the parasitic protozoa and worms (22). The intestinal parasites, especially the protozoa, are of most interest here.

The human intestinal tapeworm *Hymenolepis nana* was grown *in vitro* from the hatched egg or cysticercoid larva stage to the egg-producing adult stage in a complex medium containing horse serum (23). *Ascaris suum*, a roundworm found in the intestine of the pig, was cultured through several larval stages in a medium in which pig serum was the only undefined constituent (24). The intestinal protozoa *Entameba histolytica* of man, *Trichomonas hominis* of man and *Balantidium coli* of the pig have all been cultured in serum-containing media (25,26,27 respectively).

2. Nature of serum.

Serum is composed of many different structural and functional entities: lipoproteins, glycoproteins, enzymes and hormones, transporting substances, antibodies and enzyme inhibitors, as well as

molecules of unknown structure and function. A complex mixture like this can function any of many different ways in a growth supporting medium. Some of these different ways are discussed in section 3; and some of the serum components responsible for growth are discussed in section 4.

3. Effects of serum.

a. Metabolic effects.

A common function ascribed to serum in mammalian cell culture has been that of initiating DNA synthesis (28,29,30,31,32,33). Cells transformed by virus, however, can initiate DNA synthesis in the absence of serum, but cannot undergo mitosis without it (30,31). So, serum is involved with the DNA-synthetic and mitotic aspects of division in mammalian cell culture systems.

There are substances in serum, however, which can inhibit cell division. Human lymphocyte transformation, a process which includes cell division, stimulated by specific antigen or phytohemagglutinin (PHA) can be inhibited by an alpha globulin isolated from human serum (34). A strain of ameba of the genus Acanthameba was stimulated to divide by addition of PHA to its growth medium. Ouchterlony double diffusion evidence suggests that there are mitotic inhibitors in the amebal growth medium which are bound and inactivated by PHA, and that these inhibitors are also present in serum (35).

Serum has been shown to stimulate acid phosphatase activity (36) and delay poliovirus infection (37) in HeLa cells; induce cholesterol excretion (38) and depress lipid synthesis (39) in mouse L cells; stimulate amino acid and sulfate ion uptake in cultured chick cartilage (40); and reverse hydrocortisone-induced inhibition of glucose utilization in organ culture of chick duodenum (41).

In the late 1950's a "protein flattening factor" was shown to be present in serum (42,43). This factor was responsible for causing mammalian cells to attach to the glass surface of culture dishes. If the cells did not attach, they did not grow. Holmes, more recently, has suggested that serum not only induces cells to stick to culture dish surfaces, but also to each other (44).

b. Immunochemical effects.

Leoptospira pomona, group A streptococci, Trypanosoma theilerei and Trichomonas vaginalis all have been shown to produce certain antigens when grown in medium with serum which they did not produce when grown in medium without serum (45,46,47,48 respectively). Even though the trichomonads, which were grown in medium with horse serum, were washed 10 times they still elicited a large antibody response (in rabbits) in common with horse serum itself (48). Extensive washing of serum-grown trypanosomes was also ineffective in removing antigens common with serum (47).

4. Components of serum contributing to growth.

a. Macromolecules

Albumin has been identified as part of the contribution of serum to the growth of several mammalian tissue culture systems including Chinese hamster cells (42), mouse L cells (49), human HeLa cells (50), and monkey heart (50) and kidney (51) cells. Albumin has also been shown to contribute to the growth of Leptospira pomona (21), Rickettsia quintana (52), Bordetella pertussis (53) and several species of the flagellated protozoan parasite Trichomonas (54).

It is possible to use the iron-binding protein transferrin in place of whole serum for induction of DNA synthesis in mouse spleen cell culture (32).

An alpha-1 protein, isolated from human serum, replaces serum in support of continued growth of human HeLa, conjunctiva and heart cells (44). The alpha protein fetuin was necessary for growth of several kinds of mammalian cells (42,43,55). Cohn fraction IV, an ethanol-precipitated alpha protein-containing fraction of serum has been implicated in the growth of the spirochaete Treponema dentium (56).

Gamma globulin was selectively engulfed and degraded from the growth medium by Entameba histolytica (57). Cohn fraction II stimulated mouse 3T3 cells to initiate DNA synthesis. Cohn fraction II includes the gamma globulins; and that portion of serum that stimulated cell growth traveled with 7S gamma globulin

in a G-200 Sephadex column. However, agammaglobulinemic serum also stimulated DNA synthesis. So, a substance with properties similar to gamma globulin but not gamma globulin was responsible for stimulation of DNA synthesis in cultured mouse 3T3 cells (28).

The protein hormone insulin has been shown to be part of the serum contribution to growth in two different mammalian cell culture systems (18,31). Holley and Kiernan have reported that commercial preparations of either thyrotropic hormone or chorionic gonadotropin can replace serum for support of growth of mouse 3T3 cells in culture (58). However, the activity was due to a contaminant of the preparations which was in the molecular weight range of 100,000 daltons. The growth supporting activity of the factor was destroyed by pronase treatment. The active portion of serum was also approximately 100,000 daltons, and it too was susceptible to destruction by the proteolytic enzyme pronase (58).

In two other mammalian tissue culture systems proteolytic enzymes themselves can substitute for serum in stimulating the initiation of DNA synthesis (33,59). Trypsin and pronase were used in very low concentrations in both cases.

b. Low molecular weight substances.

Cholesterol has been shown to be part of the serum contribution to the growth of HeLa cells (60), Entameba histolytica (61), several species of Trichomonas (54), Treponema pallidum (62), Mycoplasma strain Y (63) and Histomonas meleagridis (64) a

flagellated protozoan which causes liver disease in turkeys.

Tween 80, a mixture which includes oleic, palmitic, myristic and linoleic acids, was used along with cholesterol for the growth of T. pallidum (62) and the trichomonads (54); palmitic acid was used in the H. meleagridis system (64).

A synthetic triglyceride with palmitic and oleic acids was not only a source of fatty acids for Mycoplasma strain Y, but was also necessary for dispersing cholesterol in the growth medium (63).

Particular fatty acids without cholesterol have been identified as part of the serum contribution to growth of several culture systems. Leptospira pomona (65) and monkey kidney cells (51) grew in medium supplemented with oleic acid; Hexamita inflata (14) and Chinese hamster cells (66) grew in medium supplemented with linoleic acid; and Plasmodium knowlesi (67) and the hemoflagellate Trypanosoma cruzi (68) grew in medium supplemented with stearic acid. H. inflata could also grow on oleic, stearic or linolenic acids, but linoleic acid gave the best growth (14).

5. Methods of isolating growth promoting serum fractions.

a. Examples in tissue culture systems.

1. Fischer, Puck and Sato grew HeLa cells in a mixture of amino acids, vitamins, salts, glucose and serum (60). It was found that sera from different animals, although all supporting growth, did so in different degrees. The most striking difference

was between foetal calf serum and calf serum; the former was 10 times as effective as the latter. A major difference between the two sera was known to be in the quantity of the alpha protein fetuin; foetal calf serum has more than calf serum. Fetuin was isolated from foetal calf serum by ammonium sulfate precipitation, and along with albumin could replace serum (42). Subsequently, a new growth medium was developed consisting of amino acids, choline, hypoxanthine, inositol, folic acid, B vitamins, salts, glucose and the two proteins fetuin and albumin (55).

At this point the work was continued by Ham who grew Chinese hamster (CHD-3) cells in the medium described above (69). Ham first titrated all of the components of this medium for ability to support optimal growth (69). Then, with the knowledge that albumin binds fatty acids Ham first replaced it successfully with corn oil and then with linoleic acid (66). The fetuin component was replaced with a number of natural products including chick or beef embryo extracts; blood, yeast, lactalbumin or liver hydrolysates; or a digest of ox liver - none of these mixtures contained fetuin. Continuing with the ox liver digest, Ham found that the growth promoting activity was absorbed by Dowex 50 resin, but not by Dowex 1. This suggested to him an amine with no acidic groups. Air was bubbled through an alkaline solution of the ox liver digest and then through an acid trap. The growth promoting activity was found in the acid trap; this gave more support to the idea that the component was

an amine. Spermine, putrescine or spermidine could replace ox liver digest which had replaced fetuin (70). Finally, each of the components of the now completely defined medium was titrated for its ability to support optimal growth of the CHD-3 cells (71).

2. Holmes layered human serum onto a column of glass microbeads that had been conditioned with NaOH. Serum fractions were elaborated from the column by elution with water and solutions of $NaHCO_3$, and $KHCO_3$. Growth promoting activity was found in a single fraction. The fraction was dialyzed and all of the activity remained in the retentate. Acrylamide gel discontinuous electrophoresis of the fraction showed it to be homogeneous. Human HeLa, conjunctiva and heart cells grew as well in culture on this fraction as they did on whole serum (44).

3. Frank produced a fraction from foetal calf serum according to the following protocol: ammonium sulfate precipitation, carboxy methyl cellulose and hydroxyapatite column chromatography, ammonium sulfate precipitation, and Pevikon electrophoresis. This fraction was just as effective as whole serum in stimulating DNA synthesis in cultured embryonic rat cells (29).

b. Example in a bacterial culture system.

Leptospira pomona was grown in a medium containing only 10% rabbit serum in phosphate buffer. Dialysis and 50% ammonium sulfate precipitation of the serum elaborated three fractions: dialysate, 50% supernatant (designated albumin) and 50% precipitate (designated globulin). All three fractions were necessary

for growth of the spirochaete. The albumin fraction was replaced with Amberlite 1R45 resin or soluble starch; both of these have high adsorptive qualities. The globulin was replaced with egg lecithin and the dialysate with a mixture of amino acids and B vitamins (21,72).

Subsequently, these workers developed a completely defined medium that consisted of salts, asparagine, thiamine, cyanocobalamine and a mixture of Tween 60 and Tween 80 (45,73). The globulin contribution is replaced by the fatty acid portion of the Tweens, the albumin contribution by the ring structures in the Tweens (called span) which bind fatty acids and slowly release them into solution, and the dialysate contribution by asparagine, thiamine, cyanocobalamine and salts.

In another study, the Tween 60 and Tween 80 mixture was replaced with a preparation of fatty acid-poor albumin and oleic acid (65).

c. Example in a parasitic protozoan system.

The hemoflagellate Trypanosoma lewisi is a natural pathogen of the rat. The protozoan will not grow in the mouse peritoneum unless rat serum is injected along with it (74,75). Other rodent sera similarly facilitate T. lewisi growth in the mouse (76).

In further studies with this system, Greenblatt et al. fractionated rat serum by Na_2SO_4 precipitation, G-200 Sephadex column ultrafiltration and DEAE-cellulose column chromatography. They isolated an active fraction that was homogeneous by several

criteria including ultracentrifugation. However, immunoelectrophoresis of the fraction against anti-rat serum revealed several antigens. The antigenically heterogeneous fraction had a sedimentation coefficient of 7S, had very little lipid and was, at least in part, gamma globulin. Specific antisera against rat IgA and IgM showed no reaction with the fraction in immunoelectrophoretic studies. The fraction was more potent than whole rat serum in promoting in vivo parasite growth (77).

6. Earlier attempts to replace serum in the medium for Giardia.

These are the substances which were tried in place of the 25% human serum component of Giardia growth medium; none of them supported growth. This work was done by Judy Chreist and E. A. Meyer (unpublished data).

1. Horse serum 25% (v/v)
2. Calf serum 25% (v/v)
3. Rabbit serum 25 and 50% (v/v)
4. Ham's F12 medium (71) 25,50 and 75% (v/v)
5. Neuman and Tytell serumless medium (78) 25, 50 and 75% (v/v)
6. Hemoglobin 0.5% (w/v) alone or plus each of the following singly.
   a. F12 25% (v/v)
   b. NCTC-135 50% (v/v)
   c. Serumless media 25% (v/v)
   d. Lactalbumin hydrolysate 0.2% (w/v)
7. Bacto peptone 1% (w/v)

8. Proteose peptone 2% (w/v)
9. Bacto peptone, 1% plus proteose peptone, 2%
10. Dextran 0.1, 0.5 or 1.0% (w/v)
11. Reconstituted ox gall bile, 5,10,15 or 25% (v/v)
12. Rabbit bile 2.5% (w/v)
13. Rabbit bile 2.5% plus pangestin
14. Lactalbumin hydrolysate 0.02, 0.2 and 1.0%

METHODS

A. *Giardia* Culture Techniques

1. Isolation and culture maintenance.

Giardia trophozoites were obtained originally from the small intestine of rabbits using the method of Karapetyan (9). The isolated protozoa were established in monoxenic culture with *Saccharomyces cerevisiae* (1). Physical separation of *Giardia* from yeast, and axenic cultivation of the flagellate were done as described by Meyer (11).

Trophozoites were maintained in stock culture along with but physically separate from *S. cerevisiae*, with the yeast inside a dialysis bag inserted into one arm of a U-shaped glass tube (11), and the *Giardia* outside the bag. These cultures were maintained continuously by emptying the contents of the *Giardia* side of the U-tube and refilling with fresh medium at one to three day intervals. A monolayer of trophozoites remained attached to the bottom of the U-tube through each change. Cultures were periodically checked for bacterial and fungal contamination by inoculation into fluid thioglycollate medium and into *Giardia* culture medium lacking antibiotics.

Although it is possible to continuously culture this protozoan axenically, the method above was chosen for stock cultures. The trophozoites grow as well in axenic culture as in this monoxenic-like condition; but axenic cultures are more difficult to handle. The particular strain of *Giardia* used in this work was isolated in 1964, and has since been maintained in monoxenic culture.

2. Culture medium.

A medium designated M5 was used. It was prepared according to the method described for the preparation of M3 medium (11); the only difference between the two media is that M5 lacks agar. The method of preparing M5 medium is described in the Appendix.

3. Growth assay procedure.

a. Tube inoculation.

Stock organisms were inoculated into flasks of fresh media to give starting concentrations of 1 to 9 x 10^3 organisms per ml as determined by electronic counter. Eight ml amounts of the inoculated medium were promptly dispersed into 13 x 100 mm Pyrex test tubes which were stoppered with silicon plugs and incubated on their sides at 37 C. Medium was not changed during the course of an experiment.

b. Visual scoring.

Approximate numbers of trophozoites in culture tubes were determined by direct microscopic observation as follows. At points 1, 3, and 5 cm from the bottom of each culture tube, lines were drawn perpendicular to the long axis of the tube and 180° around. During incubation, tubes were positioned with their marked sides facing down. Observations were made through an inverted microscope at 150X and 315X. Along each line the field with the highest and lowest concentration of protozoa was

scored as follows:

Score	Magnification	Trophozoites per field
1	150X	1-10
2	150X	11-225
3	315X	50-100
4	315X	101-300
5	315X	Greater than 300

The six scores determined for each tube were added giving a maximum possible score of 30. Unless otherwise specified, each reading was the average of three replicate tubes.

c. Standardization of visual scoring by means of electronic cell counts.

Immediately after visual scores were assigned, numbers of protozoa in the same tubes were counted with a Coulter Model F electronic particle counter (Coulter, Hialeah, Fla.), for the purpose of developing a correlation between visual score and actual cell count. Calibration of the Model F was done against hemocytometer counts.

Because a significant proportion of Giardia trophozoites in any culture adheres to the glass, a liquid sample pipetted from an otherwise undisturbed culture tube is not representative of concentration. To remove all the cells, each tube was first emptied into a counting vial, then refilled with Isoton (Coulter) and emptied into the same vial. The tube was filled with Isoton for a second time, refrigerated at 5 C for one hour and emptied

into the vial. A known volume of the diluted suspension was counted electronically and the number of protozoa per ml of undiluted culture medium was calculated. Microscopic observation showed that no organisms remained attached to the culture tube after the last Isoton was poured out. Also, no lysis could be detected during this procedure, since test suspensions counted before and after Isoton dilution and cooling, showed no difference in cell number.

Eight curves, describing the growth of Giardia trophozoites in M5 medium, have been determined by electronic cell count. This was done to standardize visual scores. The standard correlation of visual score vs. electronic cell count is plotted as a histogram in Fig. 1. Visual scoring was the measuring technique used in later experiments.

4. Experimental procedure.

a. Primary culture.

Thirty-two ml of each medium to be used in an experiment was prepared in a 50 ml flask. The compositions of all test media were identical to M5 medium except for the serum portion. Each experiment included an M5 control and a serumless control (medium made to volume with Hanks' balanced salt solution in place of serum). The pH of all media was adjusted to 6.7 $\pm$ 0.05; this required 1 to 4 ml from the original 32 for measurement with a pH meter, depending upon the number of adjustments with 0.1 N HCl or 7 1/2% (w/v) $NaHCO_3$ necessary. After adjustment to the desired

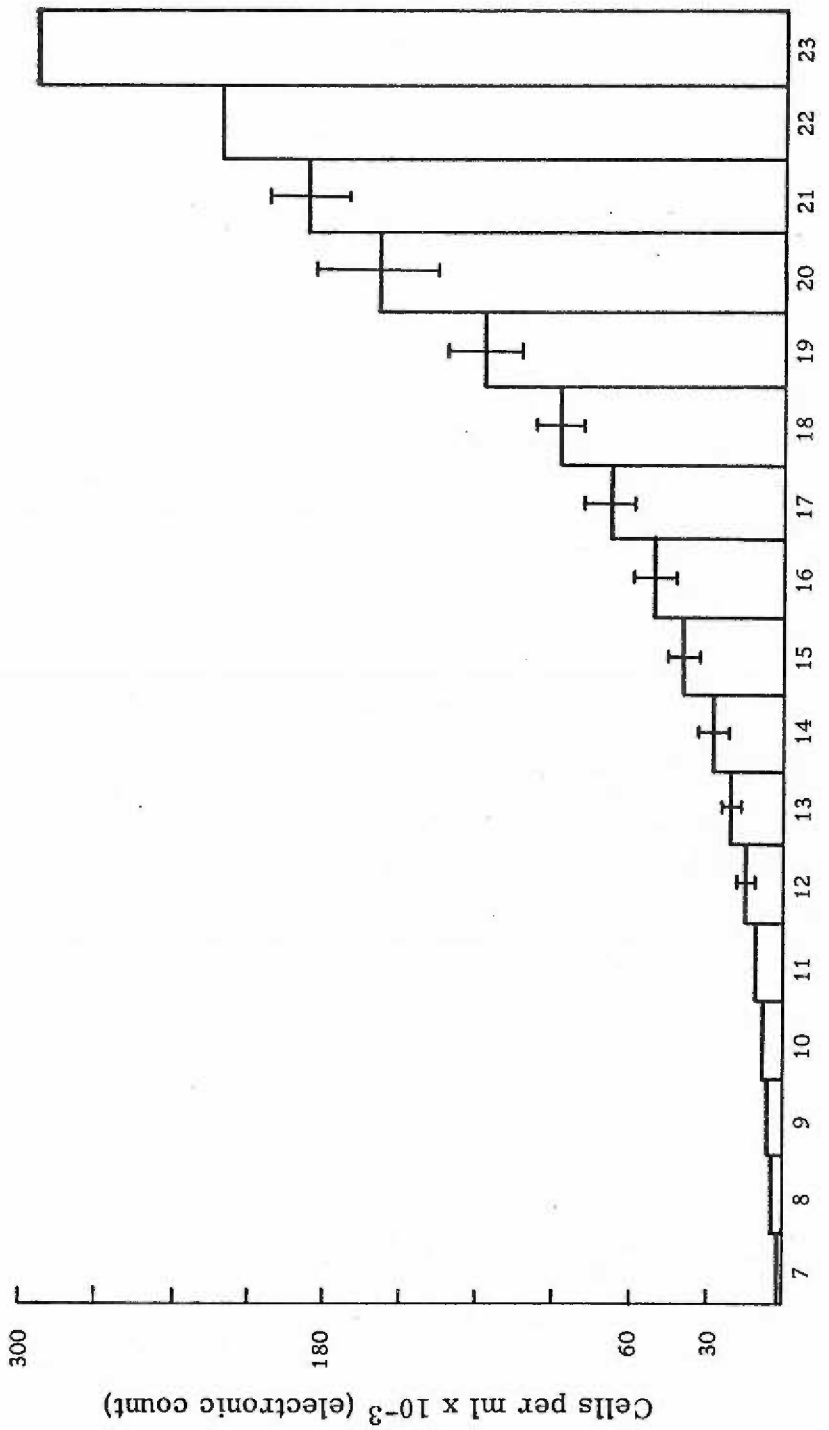


Fig. 1. Histogram of average counts for each score ± 95% confidence interval. Scores of 12 to 18 are the average of 8 measurements, scores of 19 and 20, 6 measurements and the score of 21, 4 measurements. For scores of 7 to 11, 22 and 23 there are not enough measurements to determine 95% confidence limits.

pH, all media were brought to the same volume; this varied from 28 to 31 ml in different experiments. An inoculum of 0.2 to 0.6 ml from a stock culture was pipetted into the prepared volume of each experimental medium; at the same time three Isoton blanks of the same volume were also inoculated. The blanks were counted on the electronic counter and the calculated average of the three counts considered to be the initial concentration of Giardia. The initial concentrations were between 1 and 9 x 10^3 organisms per ml; the media were dispensed into Pyrex tubes; and the tubes incubated as described in A-3-a. The number of protozoan generations occurring in each medium was calculated from initial and final concentrations. Initial concentration was determined as described above, final concentration by translation of the peak visual score into protozoa per ml. The data necessary for the translation are plotted in Fig. 1. Methods of calculating % activity and specific activity are described in the Appendix.

b. Subcultures.

The method of initiating Giardia subcultures was the same as that of initiating primary cultures except for the inoculum source, and volume employed. Here the inoculum source was organisms grown in experimental medium; the inoculum volume depended upon the concentration of cells in the inoculum source.

The initial concentration was between 1 and 9 x 10^3 protozoa per ml as before (A-4-a and A-3-a). For any new medium there was the possibility of contamination with old medium and/or the presence of intracellular pools in the transferred trophozoites. Either of these possibilities would have allowed Giardia to grow in a medium that did not supply all of its needs. Proof of the ability of a medium lacking whole serum to support Giardia growth, therefore, rests on the demonstration of continued subculture of the organisms in that medium.

B. Serum Fractionation

1. Dialysis

A known volume of serum was poured into a bag made of 15/16 inch wide dialysis tubing (Union Carbide, Chicago, Ill.); and the bag suspended in 10 volumes of distilled water. After 24 hours the water was replaced with buffered saline (bs) consisting of 0.15 M NaCl and 0.01 M Tris (2-amino-2-(hydroxy-methyl)-1-3-propanediol) (Matheson, Coleman and Bell, Norwood, Ohio). Dialysis was continued for several days with 5 more changes of bs. The distilled water dialysate was freeze-dried in a Virtis lyophilizer Model 10-010 (Virtis, Gardiner, N. J.) and the resultant dry powder dissolved in a volume of Hanks' balanced salt solution equal to the original volume of serum dialyzed. The serum retentate was harvested and its volume measured. Both fractions were sterilized by filtration through 0.45 mu pore size Millipore filter membranes in a Millipore apparatus (Millipore,

Bedford, Mass.), and frozen at -20 C. The saline dialysates were discarded.

2. Ammonium sulfate precipitation.

   a. Fifty percent of saturation.

   Thirty-eight gm of $(NH_4)_2SO_4$ powder was slowly added to 100 ml of serum at room temperature with constant mixing. The suspension was mixed in the cold for 3 hours then centrifuged at room temperature for 30 min at 2,000 g. The supernatant was decanted and the precipitate dissolved in bs. Both supernatant and dissolved precipitate were dialyzed extensively against bs at 5 C in bags made of 1 7/8 inch wide dialysis tubing (Union Carbide), then concentrated to original serum volume. Concentration was performed in an Amicon ultrafiltration cell Model 52 with a PM-10 Amicon membrane (Amicon, Lexington, Mass.). The fractions were sterilized and frozen as described in section B-1.

   b. Combination precipitations.

   The initial steps in this procedure were as described in B-2-a, viz. addition of $(NH_4)_2SO_4$ to serum, mixing, centrifugation and dissolving of the precipitate in bs. At this point, the supernatant was discarded and the dissolved precipitate mixed with varying amounts of $(NH_4)_2SO_4$ according to the following protocol: 10% was 7.6 gm of salt to 100 ml of serum and 20% was 15.4 gm to 100 ml. The remaining procedures of centrifuging, dialyzing, concentrating, sterilizing and freezing were done as in B-2-a.

3. Gel filtration.

a. Preparation of gel.

Twenty gm of dry Sephadex gel (Pharmacia, Sweden) was suspended in 1.6 liters of distilled water, boiled for 20 min with constant stirring and refined. The suspension was then allowed to swell in the cold for three days in the presence of 0.01% NaN_3. After this period the gel was equilibrated with column buffer (cb) which was 1M NaCl containing 0.1M Tris.

b. Pouring the column.

Prior to pouring, the gel suspension was degassed by vacuum. The gel was poured into the glass column (made by Pharmacia) at a pressure head of 25 cm at room temperature. After the gel had settled, the column was placed in the cold, the pressure head adjusted to approximately 15 cm, and at least one column volume (total volume of liquid and gel in poured column) of cb passed through the gel before use. A poured column was used for from 1 to 15 runs, and flushed between each run with at least one column volume of cb.

c. Preparation of sample.

Any sample to be run was dialyzed extensively against cb, brought to desired concentration by one of two methods, and refrigerated. One method of concentration is described in B-2-a, the other in B-3-e. Just before the run, the sample was made more viscous by addition of sucrose to a concentration of 10% (w/v). This was done to facilitate even entry of sample into gel.

d. Sample fractionation.

A volume of sample with sucrose was layered carefully onto the surface of the gel and washed through with cb. The column effluent was collected in a fraction collector timed to advance every 20 min. Fractions were collected in Pyrex tubes and measured at 280 mu on a Zeiss spectrophotometer (Carl Zeiss, Germany). Series of tube fractions were combined into pools according to criteria established in the Results section. From one run to the next the flow rate often decreased. To maintain the flow rate the pressure head was raised. When the flow rate dropped to less than 10 ml/hr at a pressure head of 22 cm the column was repoured.

e. Preparation of fractions for experimentation.

Large pools combined from several runs were concentrated in 8/32 inch wide Visking tubing (HMC, 52 Gloucester Place W.1, England) in a special apparatus described by Rigas (79). Low molecular weight material was drawn through the membrane by vacuum controlled with a manometer (6.25 $\pm$ 0.75 cm Hg) while large molecular weight material was held back. After concentration each sample was dialyzed extensively against bs, brought to original serum volume, then sterilized and frozen as described in B-1.

f. Aspects of separations with G-200 Sephadex.

The sample volume was 10 ml. The column diameter was 2.5 cm and the bed height 88 to 96 cm. Flow rate was 10 to 18 ml/hr

and the void volume 110 to 170 ml. Approximate column volume was 500 ml.

g. Aspects of separations with G-100 Sephadex.

The sample volume was 5 ml, the column diameter was 2.5 cm and the bed height 37 cm. The flow rate was 18 ml/hr, the void volume 70 ml and the column volume approximately 200 ml.

4. Membrane ultrafiltration.

Fifty ml of serum was put into an Amicon #52 ultrafiltration cell and forced through an Amicon XM-100 membrane at 40 psi with compressed N_2. When 35 ml had been filtered, the 15 ml remaining in the cell was brought to 50 ml with bs. The process was repeated two more times. The first ultrafiltrate and the final retentate were each made to original volume (50 ml) with bs and prepared for experimentation as in B-1. The other ultrafiltrate washes were not used.

5. DEAE-cellulose treatment.

DEAE-cellulose, DE 32 microgranular, was obtained from Whatman Company (England) and was prepared according to specifications described in the Whatman information leaflet that came with the gel. Twenty-five gm of gel was suspended in 400 ml of 0.5 N HCl. After 30 minutes the mixture was washed over Reeve Angel #2 filter paper in a Buchner funnel until the wash was up to pH 4. The drained gel was suspended in 400 ml of 0.5 N NaOH for 30 minutes then washed as before until the pH was between 6 and 7. The drained gel was equilibrated with 0.005 M K_2HPO_4, pH 8.2, the

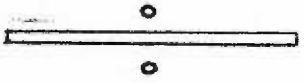
starting buffer.

The sample to be tested (G-200 Sephadex fraction II equilibrated with starting buffer) was stirred into the gel slurry. The slurry was kept in the cold with constant mixing for 1 hour. After this the gel was poured onto filter paper in a Buchner funnel and washed with 2 liters of starting buffer. All the washes were pooled and designated fraction II A. The gel was next washed with 2 liters of 0.5 M K_2HPO_4 and these washes pooled. This was designated fraction II B. The two fractions were concentrated, dialyzed against bs, made to original serum volume, sterilized and frozen as described in B-3-e. The choice of buffer and buffer molarities was based on a paper by Fahey and McLaughlin (80). The concentration of protein, determined by the Waddell method, was 5.1 mgm/ml in II A and 7.2 mgm/ml in II B. The concentration of protein in II was 12.0 mgm/ml, so there was no loss of protein with this procedure.

C. Identification and Determination of Proteins.

1. Immunoelectrophoresis.

The method used is a modification of that of Campbell et al. (81). Hot 2% ionagar No. 2 (Colab, Chicago Heights, Ill.) in veronal buffer at pH 8.6 was pipetted onto 1 inch x 3 inch glass slides at 2 ml per slide and allowed to cool. This was the pattern cut into the agar gel:



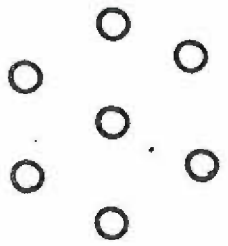
Electrophoresis of samples which had been pipetted into circular wells was carried out in an immunoelectrophoresis apparatus (National Instrument Lab., Rockville, Md.) with veronal pH 8.6 as buffer. The system was operated at 40 volts for 40 minutes.

Antiserum was pipetted into the center trough and incubation of the electrophoretically treated samples with the antiserum was done in a cold moist atmosphere for two days. After incubation, washing was done for a one day period with several changes of bs. The slides were rinsed free of salt with several quick distilled water washes and dried at room temperature for two more days. Staining was done with 1% Buffalo Black (National Aniline Division of Allied Chemical, New York, N.Y.) in a solution that was 5 parts distilled water, 5 parts methanol and 2 parts glacial acetic acid. The staining was carried on for only a few seconds. Excess stain was removed from the slides with a solution that had the same composition as the stain solvent. The destaining process was carried on until the precipitation lines were distinct.

2. Ouchterlony gel diffusion.

The method used is a modification of that of Campbell et al. (82). Ionagar slides were prepared as in section C-1. Various serum fractions, sera and antisera were pipetted into the wells cut into the slides according to the protocol for the particular experiment. The slides were incubated in a cold moist atmosphere for two days. Most of the time results were observed in unstained gels. If the gels were stained the procedures used were the same as in section C-1.

Indication of whether or not the gels were stained is made for each experiment in the Results section. The pattern cut into the gel is indicated below.



3. Discontinuous electrophoresis.

   a. E-C vertical gel method (83).

      Electrophoresis was done in an EC470 vertical gel cell (E-C Apparatus Corp., Philadelphia, Pa.) with a Buchler 3-1014A power supply (Buchler Instruments, Fort Lee, N. J.). Samples were run at 200 volts until the Bromphenol blue dye had migrated to the bottom of the spacer gel which was 4% Cyanogum (E-C Corporation) in Tris-HCl buffer at pH 6.7. Cyanogum is 95% acrylamide and 5% Bis (N, N' methylene bisacrylamide). Four hundred volts was then applied to move the sample through the running gel which were 7% Cyanogum in Tris-HCl buffer at pH 8.9. The electrode buffer was Tris-glycine at pH 8.3. The sample mixture was 5 parts of a mixture of 4% acrylamide (Matheson, Coleman and Bell) and 5% sucrose (Mallinckrodt, St. Louis, Mo.) in Tris-HCl buffer at pH 6.7 made dark blue with Bromphenol blue; and one part protein sample solution. The protein sample was

dissolved in bs; and the concentration adjusted so that the final protein concentration of the sample applied to the gel was 2 to 5 mgm/ml. The gel was stained with 1% Amido Black 10B (E-C Corporation) in 7% glacial acetic acid. Destaining was done in plain 7% glacial acetic acid and took several days with many changes of destaining solution.

b. Electrophoretic method of Davis (84).

The fractions examined by this procedure were analyzed in the laboratory of Professor Dennis Burger (Veterans Administration Hospital, Portland, Oregon). The large pore gel was 2 1/2% acrylamide (Eastman Chemical, Rochester, N. Y.) and 5/8% Bis (Eastman) in Tris-HCl buffer at pH 6.7, and the small pore gel was 11.2% acrylamide and 0.03% Bis in Tris-HCl at pH 8.9. The electrode buffer was Tris-glycine at pH 8.3. Samples were run at 2 to 5 ua per cylinder in a Buchler electrophoretic apparatus (Buchler Instruments). Staining was done with 1% Amido Schwartz (Allied Chem., Moorestown, N. J.) in 7% glacial acetic acid. Destaining was done in 7% glacial acetic acid.

4. Waddell method (85) for protein determination.

All protein samples were diluted with bs. Murphy and Kies (86) have shown that the concentrations of NaCl and Tris in bs do not affect the spectrophotometric readings at the wavelengths used. Optical density was measured on a Zeiss Spectroscope (Carl Zeiss) at 215 and 225 mu wavelength, and the difference calculated.

Increasing concentrations of human Cohn Fraction V (Nut. Bioch. Co. Cleveland, Ohio) from 20 to 80 ug/ml were measured in this manner and a standard curve developed. All unknown concentrations were determined by referring to this standard curve.

5. Radial diffusion method for quantitation of specific proteins.

This method is a modification of that described by Mancini et al (87). It was performed in Professor Bernard Pirofsky's laboratory (Div. Allergy and Infectious Disease, U. of Ore. Med. Sch.). Standard agar plates and reference serums were obtained from Hyland Laboratories (Los Angeles, Calif.). Several known concentrations of each antigen were pipetted into the wells in the agar plates. Specific antibody had been distributed evenly throughout the agar. After incubation, rings of precipitation were formed around each well and the diameter of each ring measured. Each diameter was plotted against its respective concentration and a standard curve was drawn. Each unknown serum was run along with the reference serums and its precipitation ring diameter translated into concentration on the basis of the standard curve. Determinations were done for human IgA, IgG and IgM.

MATERIALS

A. Serum for Routine Culture

Human serum for *Giardia* culture medium was obtained from three sources: University of Oregon Medical School Hospital (CP), St. Vincent's Hospital in Portland, Oregon (SV) and the Oregon State Public Health Service in Portland (PH). Pools were generally 300 to 1,000 ml with the exception of No. 30 which was 7 liters. Each pool was noted by number and source. All sera were less than 2 months old (storage at -20 C) at use except No. 30 which was used for one year.

B. Special Sera

Sera from patients with various diseases was obtained from Professor Bernard Pirofsky, Division of Allergy and Infectious Disease and Professor Fred Hecht (Experimental Medicine) both of the University of Oregon Medical School.

C. Commercial Preparations

1. Antisera

a. Hyland Laboratories, Los Angeles, California

Goat anti-human IgA (α-chain specific)

Goat anti-human IgG

Goat anti-human Transferrin

Goat anti-human Ceruloplasmin

Goat anti-human α 2 macroglobulin

Horse anti-human crystallized albumin

b. Kallestad Laboratories, Minneapolis, Minn.

Horse anti-human haptoglobin.

c. Hoechst Pharmaceutical Co., Kansas City, Mo.

Rabbit anti-human hemopexin

Rabbit anti-human β-lipoprotein

Rabbit anti-human α1-antitrypsin

Horse anti-human Gc globulin

RESULTS

A. Development of the Growth Assay System

The growth medium for Giardia trophozoites contains Hanks' balanced salt solution (88), human serum, yeast extract, the synthetic tissue culture medium NCTC-135 (89), a solution of the reducing agents cysteine and glutathione, and antibiotics. Hanks' solution serves as an isotonic vehicle for the other constituents in the medium.

Prior to this work, Giardia growth response to varying concentrations of the yeast extract and NCTC-135 components, was determined by E. A. Meyer (unpublished data). Since the concentration of both components could be increased with no increase in growth response they were considered to be in excess in the M5 growth medium. The optimal concentration of reducing agents and the optimal pH were also determined by Meyer (11).

The first step of this work was to determine the effect of varying the serum concentration in M5 medium on Giardia growth. Fig. 2 shows that the protozoa grow to increasingly higher concentrations as the amount of serum in the medium increases. Translating the visual scores in Fig. 2 into cells per ml, it can be seen that 5, 10, 15, 20 and 25% serum levels in M5 produce peak protozoan concentrations of 60, 90, 130, 160 and 190 x 10^3 cells per ml respectively. This is an increase of approximately 30 x 10^3 Giardia per ml for each 5% increase in serum. At 50% serum the peak concentration is 220 x 10^3 cells per ml; an increase of only 40 x 10^3 cells per ml for a 25% increase in serum. Above the 25% serum level, the rate of increase in peak concentrations of protozoa decreases with increasing serum concentration in the growth medium.

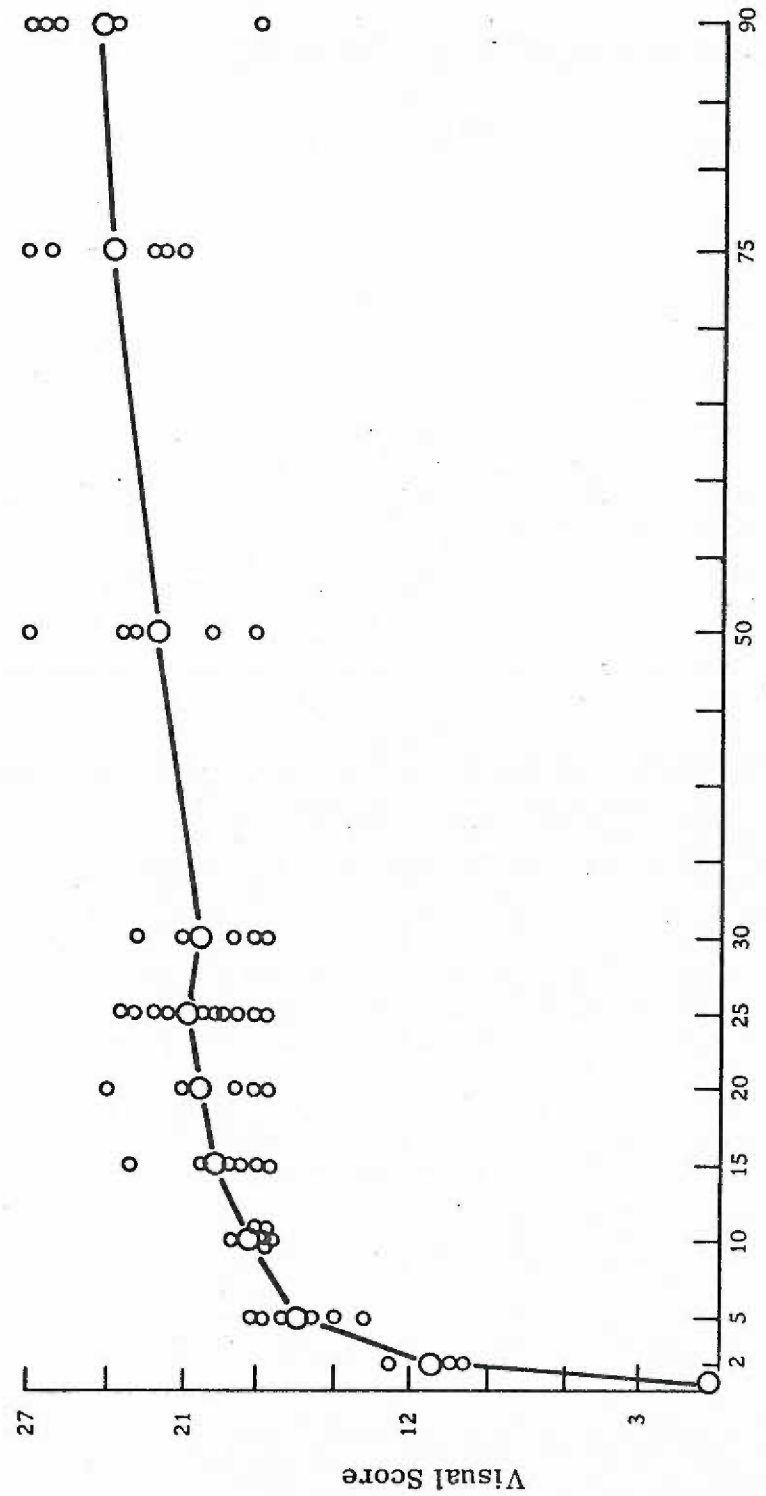


Fig. 2. Growth response to varying serum concentrations in M5 medium. As the % by volume of serum increases the % of Hanks' balanced salt solution decreases; other components of the medium are unchanged. Each point is a separate experiment; the minimum number of trials for any serum concentration was 4. Ninety % was the maximum volume of serum that was used, because of the necessity for 5% NCTC-135 and 5% reducing solution (see appendix).

Twenty-five percent was the level of serum in M5 chosen to be used for all experiments to follow. There were two reasons for this: first, because Giardia grew to higher concentrations in medium with this level of serum than in medium with lower serum levels, and second, because Giardia made more efficient use of serum at this level than at higher levels.

A typical curve representing the growth of Giardia in M5 medium containing 25% serum is shown in Fig. 3. The solid line was made with protozoan concentration values determined by electronic counting; it shows typical lag, exponential and stationary phases of growth. The visual score (dashed line) values can be seen to describe exponential phase accurately, but decrease during stationary phase when the electronically-counted curve is leveling. This is because the trophozoites no longer adhere to the surface of the culture tube during this period. Consequently, visual scoring is not an accurate means of estimating concentration of protozoa during stationary phase. For this reason the correlation of visual score and electronic cell count shown in Fig. 1 (Methods section) was done only during exponential growth. For the purpose of comparing Giardia growth in medium containing whole serum with growth in medium containing different serum fractions or substitutes this correlation was adequate, since the number of generations per culture (the criterion for comparison) was determined from the peak exponential phase concentration and the zero-time concentration of protozoa per ml. The zero-time or initial concentration of Giardia was determined by electronic cell count as described in Methods section A-3-a.

Table 1 shows data from 8 growth curves determined in M5 medium with 5 different pools of serum. The average number of generations per culture was 5.5 ± 0.3 and the average generation time 18.1 ± 1.6 hr. Generation

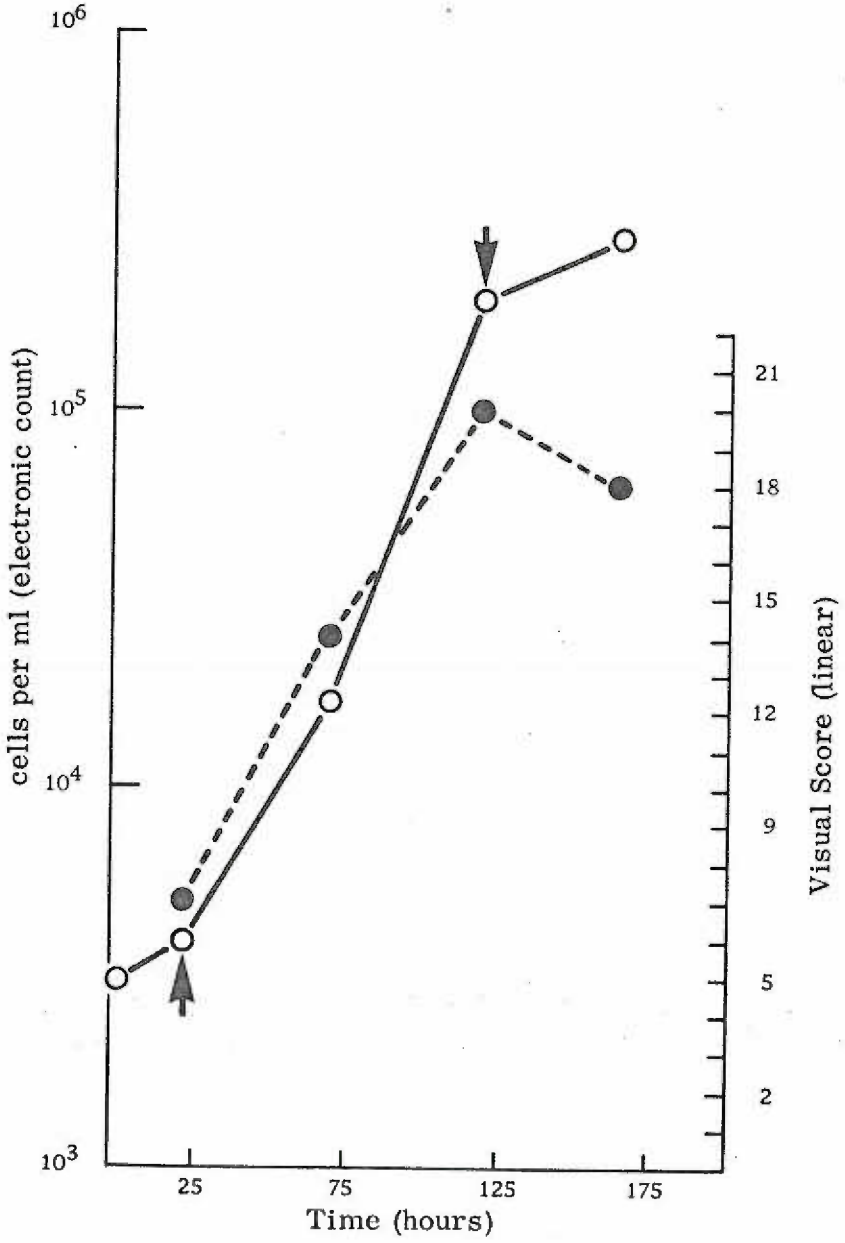


Fig. 3. Growth curve of Giardia in M5 medium. Each point is the average of three tubes. The solid line is the electronic count, and the dotted line visual score. Arrows denote that part of the solid curve used to determine generation times.

Table 1. Growth curve data determined with electronic counter. Generation times are determined between first day reading and peak of log reading.

Cells per ml x 10^{-3}			Number of Generations	Generation Time in hours	Serum Pool
Initial	First Day Reading (hours)	Peak of Log Growth (hours)			
4.4	9.0 (22)	184 (121)	5.4	22.7	17 SVPH[1]
5.0	9.7 (25)	290 (92)	5.8	13.8	22 SVPH
6.6	11.7 (20)	91 (67)	3.9	15.9	23 SVPH
2.1	4.8 (17)	88 (90)	5.4	17.4	23 SVPH
4.0	6.5 (26)	192 (95)	5.5	14.1	23 SVPH
2.1	4.1 (24)	209 (117)	6.5	16.3	24 PH[2]
3.2	4.1 (23)	198 (120)	5.6	17.4	24 PH
1.7	5.2 (25)	181 (163)	6.2	26.9	28 SVPH

1. Pool of serum from St. Vincent's Hospital and Public Health Service
2. Pool of serum from Public Health Service

time was determined from the beginning of exponential phase to the peak as is denoted by the position of the arrows in Fig. 3.

Complement was routinely inactivated by heating serum at 56 C for 30 minutes, but preliminary experiments showed that Giardia grew just as well in medium containing untreated serum. Freezing and thawing of serum 1, 2 and 4 times resulted in no deleterious effect on its Giardia growth promoting activity. Heating serum in a water bath for 2 hours at 40, 55 and 65 C had no deleterious effect on its ability to promote Giardia growth. At 70 C the serum coagulated very rapidly, and could not be tested in the liquid M5 medium.

B. Attempts to Replace Whole Serum with Serum Fractions Prepared by Different Methods

1. Fractions prepared by dialysis.

Two fractions were prepared from extensive dialysis of serum: a retentate which was the material that remained inside the membrane bag and a dialysate which was the material that came through the bag in the first volume of dialyzing liquid. Each fraction alone and both fractions together (reconstituted) were incorporated into M5 medium in place of whole serum and tested for their growth promoting activity. Table 2 shows the results of these experiments. The retentate had an activity of 99 $\pm$ 5% of control, the dialysate 0% and the reconstituted preparation 101 $\pm$ 1%. There was no significant difference between the retentate and the reconstituted preparation, each of which had the same activity as the whole serum control. The three experiments were done with three different pools of serum; there was no major difference among them.

Table 2. Growth response to dialyzed serum fractions in M5 medium

Fraction	Serum Pool			Ave ± S.D.
	8 SVPH	10-PH	9-SV	
Whole Serum Control	100	100	100	--
No Serum	0	0	0	0
Dialysate	0	0	0	0
Retentate	94	108	95	99 ± 5
Reconstituted	103	100	100	101 ± 1

Table 3. Growth response to fractions from 50% ammonium sulfate precipitation in M5 medium expressed as % of control activity

Fraction	Serum Pool						Ave ± S.D.
	11 PH	14 SVPH	22-SVPH	22-SVPH	23-SVPH	24-PH	
Whole Serum Control	100	100	100	100	100	100	--
No Serum	0	0	0	0	0	0	0
Supernate	0	0	0	0	0	0	0
Precipitate	90	92	107	103	121	106	103 ± 5
Reconstituted	65	100	119	100	128	100	102 ± 9

2. Fractions prepared by ammonium sulfate precipitation.

The two fractions prepared from serum treated with 50% of saturation ammonium sulfate were tested for their ability to support Giardia growth in M5 medium. Also, a reconstituted preparation was made and tested. The results are shown in Table 3. The precipitate had an activity of 103 ± 5% of control, the supernatant 0% and the reconstituted preparation 102 ± 9%. There was no significant difference between the precipitate, the reconstituted preparation, and the whole serum control. The 6 experiments were done with 5 different pools of serum. The precipitate and reconstituted preparations from serum pool 11 gave depressed responses of 90 and 65% of control activity respectively, and these two preparations from serum pool 23 gave elevated responses of 121 and 128% of control respectively. The activities of these two preparations from the other three serum pools were between these extremes. None of the supernatant preparations had any activity.

The 50% precipitate from serum pool 24, which had an activity of 106% of control (Table 3), was dissolved in buffered saline (bs) and treated with 10% of saturation ammonium sulfate. Table 4 shows that the 0 to 10% ammonium sulfate-precipitated fraction had no growth promoting activity when substituted for whole serum in M5; that the 10 to 50% fraction had 73% of control activity and that the two fractions together had 87% of control activity. So, the second ammonium sulfate treatment resulted in a loss in total activity of approximately 20% (106 minus 87%). The major activity was in the

Table 4. Growth response to fractions from combination ammonium sulfate precipitation in M5 medium expressed as % of control activity

Serum Pool	Serum Preparation				
	Wh. Serum Control	No Serum	0-10%	10-50%	0-10% + 10-50%
24-PH	100	0	0	73	87

Table 5. Growth response to fractions from combination ammonium sulfate precipitation in M5 medium expressed as % of control activity

Serum Pool	Serum Preparation				
	Wh. Serum Control	No Serum	20 P (0-20%)	20 Su (20-50%)	0-20% + 20-50%
23-SVPH	100	0	61	-	90
24-PH	100	0	48	0	84
24-PH	100	0	71	0	83

10 to 50% fraction, but the 0 to 10% did have complimentary activity even though it supported no growth by itself.

Fifty percent precipitates from serum pools 23 and 24 (Table 3), which had an average activity of 113% of control, were dissolved in bs as before, and now treated with 20% of saturation ammonium sulfate. Table 5 shows results of experiments done with fractions prepared by this procedure. The major growth promoting activity now moved to the precipitate, or 0 to 20% fraction, and averaged 60 $\pm$ 7% of control for the 3 experiments. The 20 to 50% fraction when tested as the sole serum component of the medium had no activity, but when tested in combination with the 0 to 20% fraction, activity increased to 86 $\pm$ 2% of control. Again there was a loss in total activity due to the second precipitation.

At this point the 0 to 20% fraction (20P) and the 20 to 50% fraction (20Su) were filtered through a G-200 Sephadex column. Fig. 4 shows the 280 mu optical density tracings of these fractions. The 7S peak is noticeably absent from the 20Su tracing which had no growth promoting activity by itself. The 4S peak is very small in the 20P fraction and very large in the 20Su fraction; and the 20Su fraction enhanced the activity of the 20P fraction.

Further serum fractionation studies using repeated ammonium sulfate treatments were not pursued since this procedure consistently resulted in a reduction of growth promoting activity.

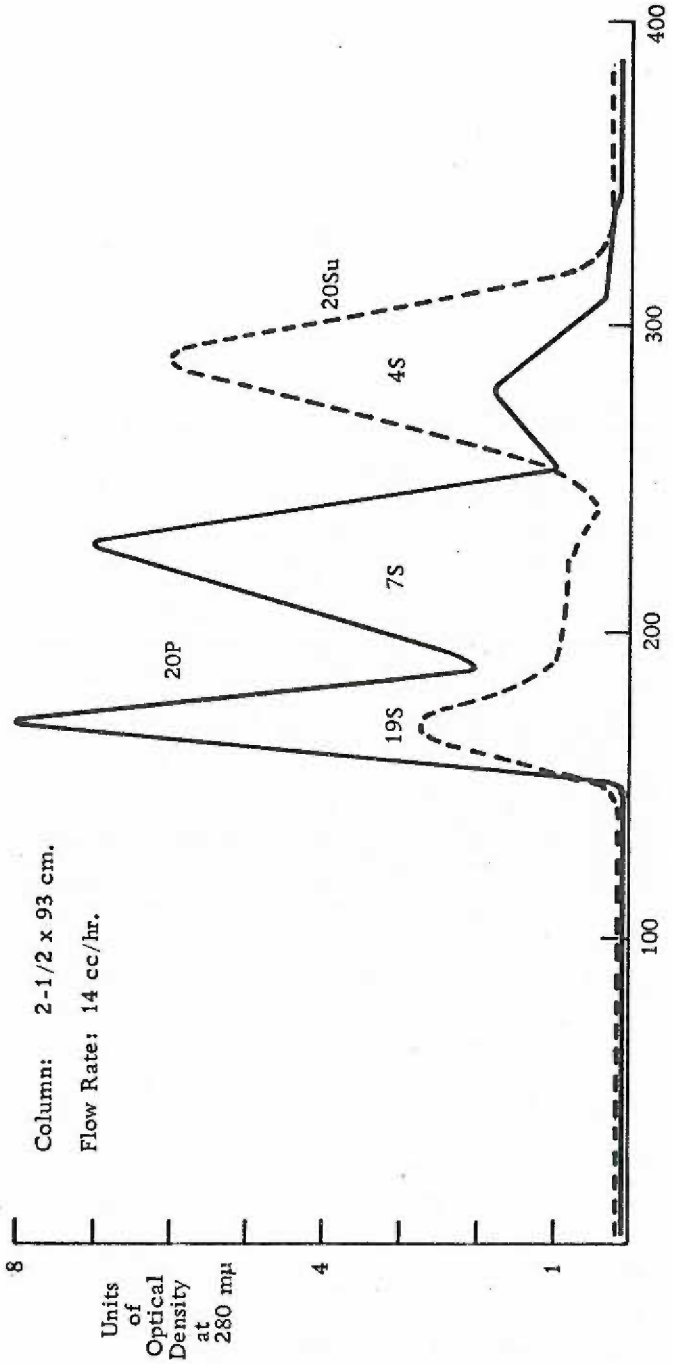


Fig. 4. G-200 Sephadex ultrafiltration of ammonium sulfate-precipitated fractions. Dotted line is 20-50% fraction and solid line is 0-20% fraction.

3. Fractions prepared by membrane ultrafiltration.

Ultrafiltration across a membrane that holds back molecules of molecular weight greater than 100,000 was done on whole serum. The material that did not pass through the membrane (retentate) was washed several times with bs, and refiltered after each wash. The purpose of this method was to separate the 4S peak from the rest of serum, so its importance to Giardia growth could be determined.

Fig. 5 shows G-200 Sephadex tracings of the retentate and ultrafiltrate prepared by this method. Material in the 19S, 7S and 4S size ranges is present in both fractions. Since the ultrafiltration method did not adequately produce a separation of the 4S material from the rest of serum, it was not pursued.

4. Fractions prepared by G-200 Sephadex gel filtration-three fraction method.

G-200 Sephadex, used heretofore only as a descriptive tool for fractions prepared in other ways, was now employed as a preparative tool. Fig. 6 shows a tracing of 280 mu-absorbing material as it is eluted from the gel column. Three fractions were collected from the original 50% ammonium sulfate precipitate that was layered onto the column. The 19S peak was designated fraction I, the 7S peak fraction II and the 4S peak fraction III.

Table 6 shows results of experiments done with these 3 fractions prepared from 4 different pools of serum. Fraction III never supported growth when substituted for whole serum in M5 medium; fraction II + III always did (75 $\pm$ 7% of control activity) and the

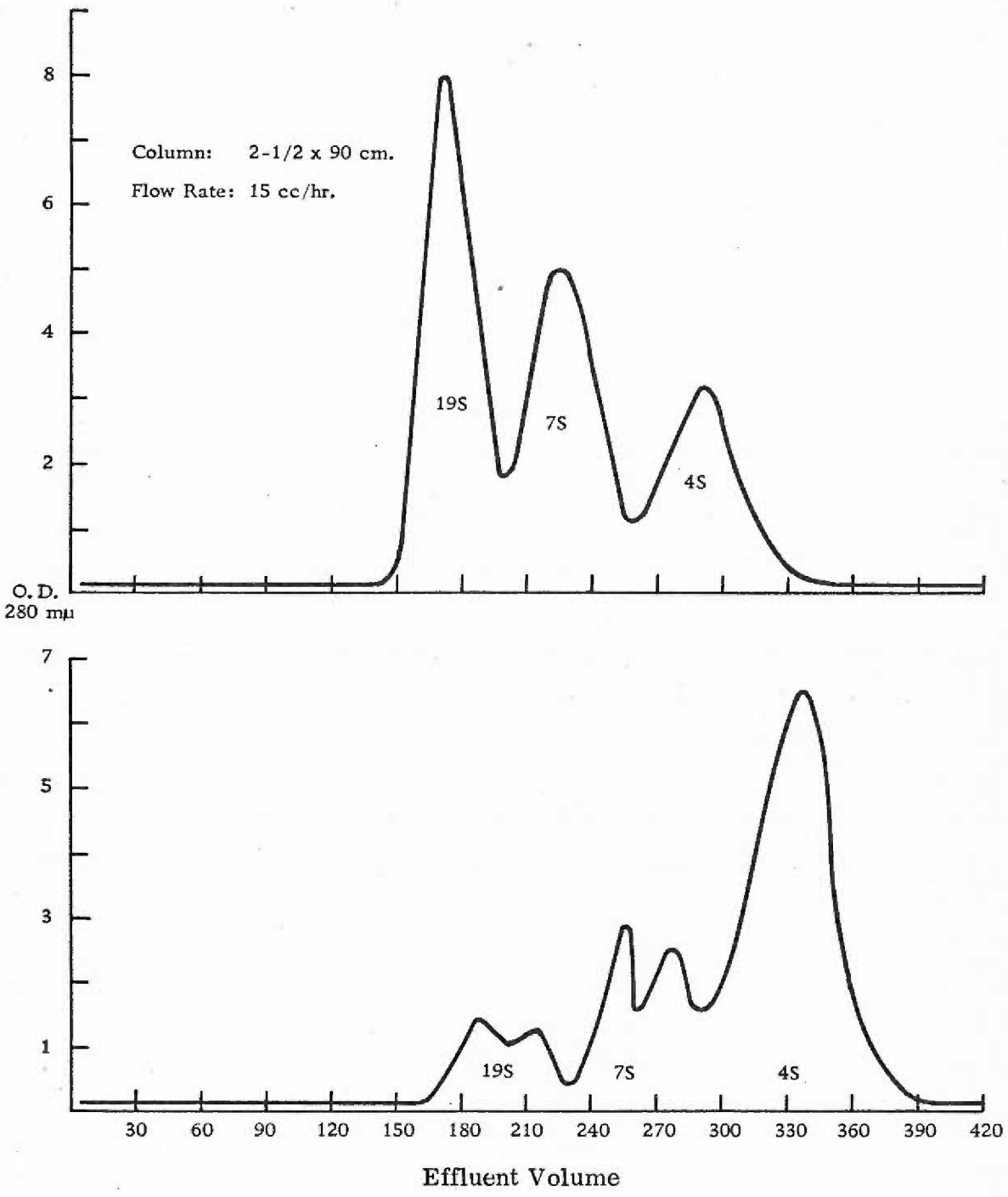


Fig. 5. G-200 Sephadex ultrafiltration pattern of Amicon ultrafiltration fractions. Top: retentate, bottom: ultrafiltrate.

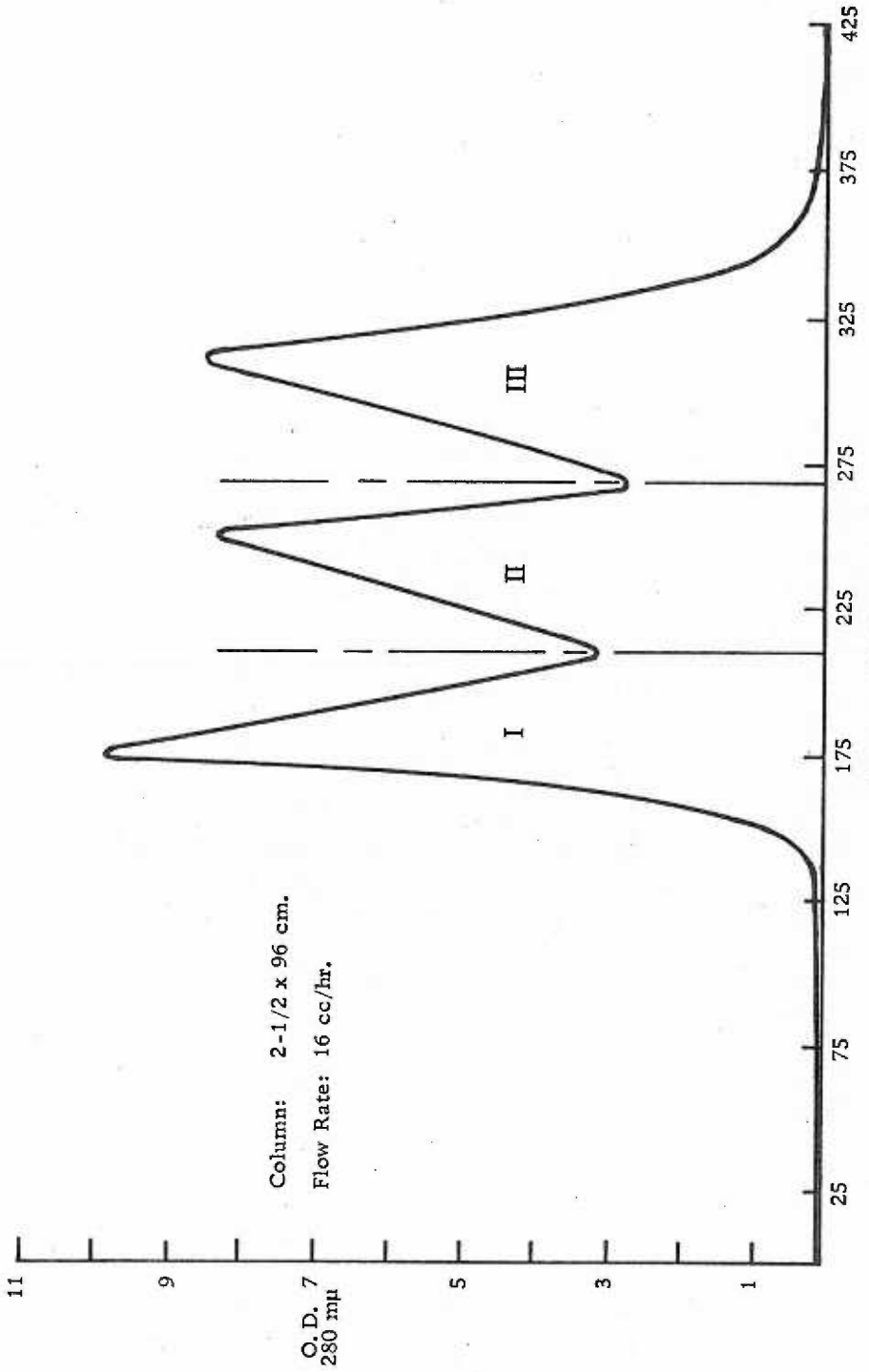


Fig. 6. G-200 Sephadex ultrafiltration pattern of 50% ammonium sulfate precipitate. The 19S peak is designated I, the 7S peak II, and the 4S peak III.

Table 6. Growth response to G-200 Sephadex fractions in M5 medium expressed as % of control activity

Serum Pool	Serum Preparation								
	Wh. Serum Control	No Serum	I	II	III	I + II	I + III	II + III	I + II + III
22-SVPH	100	0	23	83	0	--	--	--	97
	100	0	--	77	-	77	91	91	107
23-SVPH	100	0	12	88	0	100	35	100	107
	100	0	31	73	0	86	78	93	93
24-PH	100	0	0	10	0	0	15	56	56
	100	0	0	22	0	0	0	65	78
28-SVPH	100	0	0	0	0	0	0	52	52

reconstituted 50% precipitate, I + II + III always did (84 ± 8%). Fraction II from serum pools 22 and 23 had an average activity of 80 ± 3% of control for 4 experiments, but from serum pools 24 and 28 fraction II had an average activity of 11 ± 6% for 3 experiments. Other preparations from serum pools 24 and 28 also gave depressed growth responses. Fraction I from serum pools 24 and 28 had no activity, but from serum pools 22 and 23 had an average activity of 22 ± 5% of control.

An additional serum pool (29SV) was fractionated and tested in growth medium as before. Table 7 shows growth data for the preparations II, III and II + III from all 5 serum pools now tested (a total of 10 experiments). Again fraction III alone in place of whole serum in M5 never had any growth promoting activity; the combination of II + III always had activity and averaged 75 ± 5% of control; and fraction II had erratic activity. The fraction II preparations from serum pools 22 and 23 had consistently high activities (77, 88 and 73%), from serum pool 24 consistently low (10, 22 and 0%) and from serum pool 28 irregular activities (0, 52 and 16%).

Subculture of Giardia was attempted using the combination of fractions II + III to further check its adequacy as a whole serum replacement. Up to this point, all experiments were initiated with Giardia grown in stock culture. Fig. 7 shows that the Giardia which grew initially in M5 containing II + III in place of serum, grew again and to the same peak concentration when subcultured.

Table 7. Collected data from experiments with G-200 Sephadex fractions II, III and II + III in M5 medium. Growth is expressed as % of control activity.

Serum Pool	Serum Preparation				
	Wh. Serum Control	No Serum	II	III	II + III
22 SVPH	100	0	77	0	91
23 SVPH	100	0	88	0	100
	100	0	73	0	93
24 PH	100	0	10	0	56
	100	0	22	0	65
	100	0	0	-	70
28 SVPH	100	0	0	0	52
	100	0	52	0	79
	100	0	16	-	73
29 SV	100	0	49	0	75

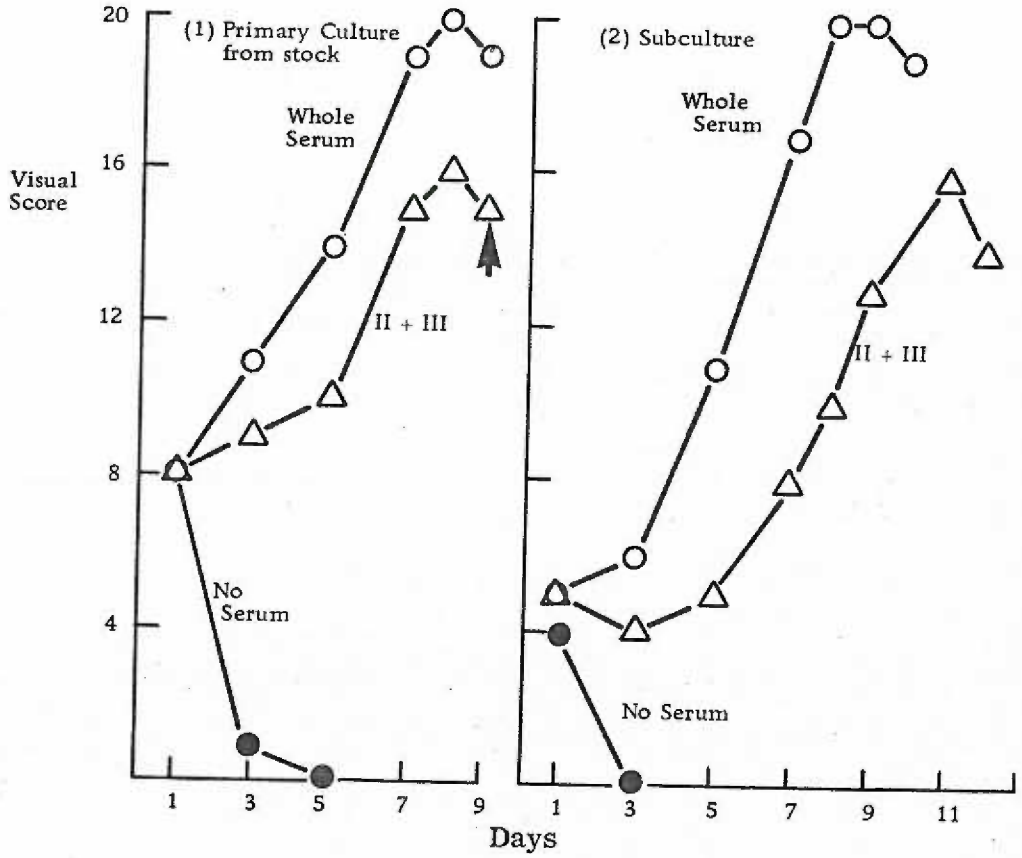


Fig. 7. Growth response in primary culture and subculture to fraction II + III in M5 medium. Each point is the average of three replicate cultures. Serum pool is 24. Standard deviation is ± 1 for every point. Arrow indicates point at which II + III cells were harvested and inoculated into each of the variables in (2).

C. Attempts to Replace G-200 Sephadex Fractions II and III.

1. G-100 Sephadex gel filtration of II and III.

Fig. 8 shows the 280 mu absorption patterns of the effluents from column runs of fraction II and fraction III. A single symmetrical peak occurred in each case. Since this method provided no means for subdividing either fraction, it was not used further.

2. Attempt to replace III with the supernatant (S) from 50% ammonium sulfate precipitation.

Serum pool 28 was treated with 50% of saturation ammonium sulfate. The precipitate was passed through G-200 Sephadex. There were 4 resultant serum fractions: the supernatant from ammonium sulfate treatment (S) and the three Sephadex fractions of the 50% precipitate - I, II and III. Table 8 shows that in 3 experiments, II + S in place of whole serum has an activity (55 $\pm$ 8% of control) approximately equal to that of II + III (61 $\pm$ 10%).

In Table 8 there is only one control of medium with fraction III alone, and only one with fraction S alone. However, Table 7 shows 8 experiments where fraction III in M5 had no activity in the absence of other serum constituents and Table 3 shows 6 experiments where fraction S, the 50% supernatant, had no activity by itself.

3. Fractions of II prepared by DEAE-cellulose treatment.

A simple batch procedure performed on fraction II resulted in the two fractions II A and II B. Table 9 shows that either of these fractions or a reconstituted preparation of the two supported little or no growth when combined with fraction III and incorporated into

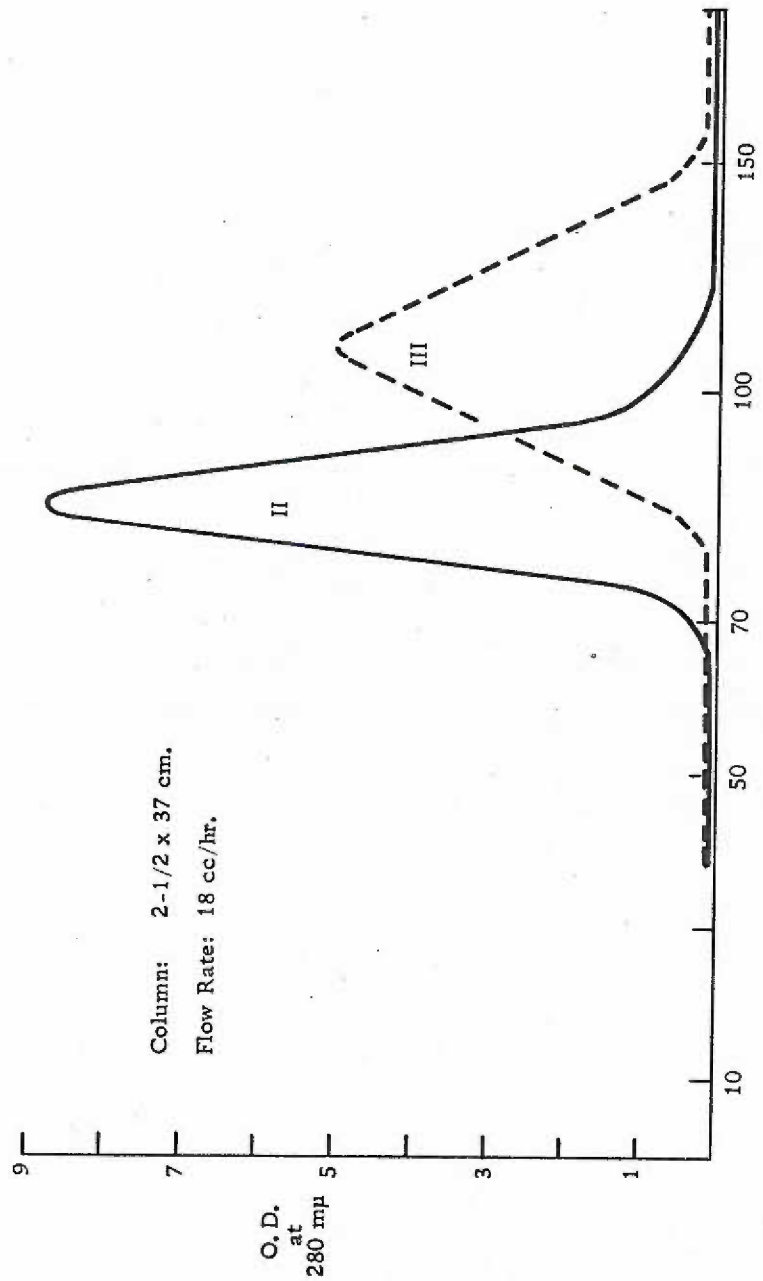


Fig. 8. G-100 Sephadex ultrafiltration pattern of G-200 Sephadex fractions II and III.

Table 8. Activity of supernatant (S) from 50% ammonium sulfate precipitation compared to fraction III from G-200 Sephadex ultrafiltration. Growth is expressed as % of control activity. Bottom line is average ± S.D. All preparations are from serum pool 28.

Serum Preparation						
Wh. Serum Control	No Serum	II	II + III	II + S	III	S
--	0	0	46*	46*	-	-
100	0	34	--	71	-	0
100	0	52	79	--	0	-
100	0	--	58	58	-	-
100	0	--	61 ± 10	55 ± 8	-	-

* These values are based on an average whole serum control value taken from all experiments with serum pool 28.

Table 9. Growth promoting activity of DEAE-cellulose fractions of G-200 Sephadex fraction II. Growth is expressed as % of control activity. Both preparations were from serum pool 28.

DEAE Preparation	Serum Fractions					
	Whole Serum	No Serum	II + III	IIA + III	IIB + III	IIA + IIB + III
1	100	0	59	0	17	21
2	100	0	53	0	0	0

M5 in place of whole serum. Untreated fraction II + fraction III had an average activity of 56% of control for the two experiments. In one case, there was some slight activity in II B (17% of control when incorporated into M5 with fraction III).

D. Attempts to Replace Whole Serum with G-200 Sephadex Fractions Prepared by the Six Fraction Method.

1. The six fractions.

Fig. 9 shows a 280 mu tracing of the effluent from a G-200 Sephadex column run of a 50% ammonium sulfate precipitate from serum pool 30. The figure also shows the separation of the material into 6 fractions which were designated 1A, 1B, 2A, 2B, 3A and 3B.

Because the same fractions from different serum pools gave different growth responses in some cases (e.g. fraction II, Table 6), a single serum pool, number 30, was used from this point on.

Table 10 shows protein concentrations of the 6 fractions from 3 different preparations of serum pool 30. There are differences in the amount of protein in a particular fraction from one preparation to the next, but the overall distribution in the 6 fractions follows a consistent pattern. The % recovery of protein from the column is remarkably consistent at 90.

2. Primary culture experiments.

Table 11 shows that either fraction 1B or 2A consistently supported Giardia growth when substituted for whole serum in M5 medium. None of the other 4 fractions had activity when substituted for serum.

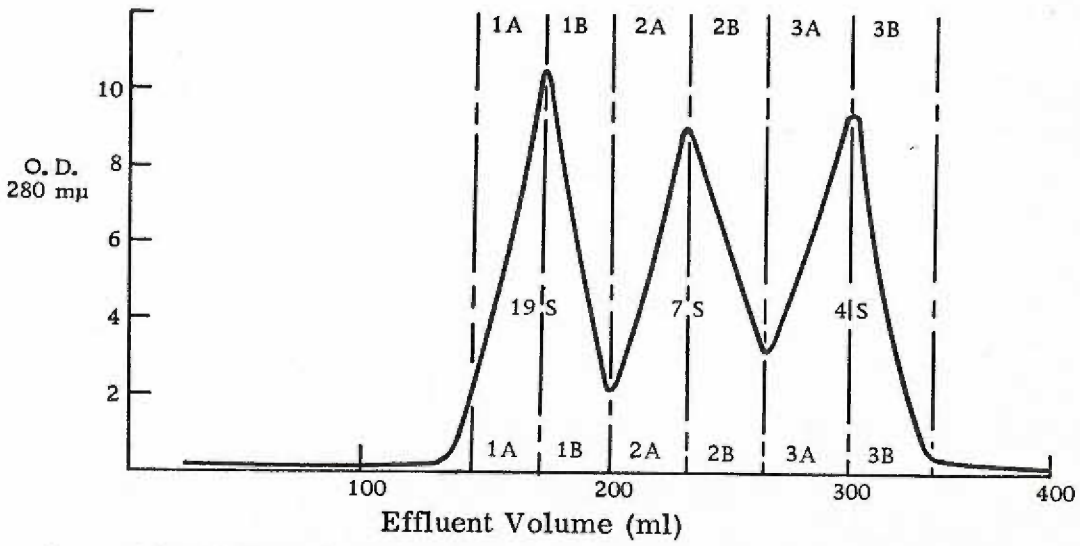


Fig. 9. G-200 Sephadex ultrafiltration pattern of 50% ammonium sulfate precipitate. Material was separated into 6 fractions according to the figure. Serum pool is 30 SVPHCP (Pool of serum from St. Vincent's Hospital, Public Health Service and University of Oregon Medical School Hospital).

Table 10. Protein concentrations of the 6 fractions in Fig. 9 , and % recovery of protein from G-200 Sephadex column. Protein concentrations were determined by the Waddell method. All preparations were from serum pool 30.

Preparation	Fraction (mgm/ml)						Total of 6 Fractions (mgm/ml)	Original 50% Precipitate (mgm/ml)	% Recovery
	1A	1B	2A	2B	3A	3B			
A	6.5	5.9	8.8	6.9	16.0	12.4	56.5	62.5	90.4
B	6.1	6.2	8.7	6.2	20.0	9.6	56.8	63.4	89.6
C	5.2	7.7	7.5	4.9	15.7	10.8	51.8	57.5	90.1

Table 11. Growth response to G-200 Sephadex fractions in M5 medium expressed as % of control activity. All fractions were prepared from serum pool 30.

Fraction	Average % Activity ± S.D.	Replicates
1A	0	5
1B	63 ± 7	8
2A	64 ± 4	16
2B	0	5
3A	0	5
3B	0	5
Reconstituted (1A+1B+2A +2B+3A+3B)	64 ± 6	5
1B+2A	89 ± 4	5
2X 1B	81 ± 4	4
2X 2A	76 ± 6	6

Adding 1B to 2A or doubling either 1B or 2A improved growth promoting activity. However, when all 6 fractions were reconstituted, the resultant activity was significantly lower than the combination of only 1B and 2A which comprises part of the reconstituted preparation.

Because fraction III had complimentary activity to fraction II (Table 7) fraction 3B was tested for complimentary activity to the 1B/2A component. Fraction 3B was selected in preference to 3A, because it was less likely to be contaminated by anything in 1B or 2A. If fraction 3B was complimentary, then a second substance (or group of substances) besides the substance (s) in 1B and 2A, would again be implicated. Table 12 shows that the combination of fractions 2A + 3B in place of whole serum in M5 medium, had a higher activity than fraction 2A alone in 4 replicate experiments (75 $\pm$ 4% vs 54 $\pm$ 4% of control). The average activity of the 4 replicates of M5 containing 2A + 3B in place of whole serum is the same as that of the 10 replicates of II + III shown in Table 7. All of the 2A + 3B preparations were made from serum pool 30, while the II + III preparations came from 5 different pools of serum.

3. Subculture experiments.

Table 13 shows the results of attempts to subculture trophozoites grown in M5 medium containing 1B, 2A or the combination of 1B and 2A in place of whole serum. In 7 of 8 attempts with the 3 different media, subcultures failed to grow. No attempt was made to further subculture those organisms which grew to a limited extent on first subculture in 2A-containing medium.

Table 12. Growth promoting activity of fraction 2A compared to 2A + 3B in M5 medium. Growth is expressed as % of control activity. All preparations from serum pool 30.

Fraction	Experiment				Average ± S.D.
	1	2	3	4	
2A	49	45	57	65	54 ± 4
2A + 3B	67	84	70	78	75 ± 4

Table 13. Growth response in primary culture and subculture to G-200 Sephadex fractions in M5 medium. At peak of primary growth, organisms were subcultured into the fractions indicated. WS is whole serum control; NG is no growth. All preparations were from serum pool 30.

Primary Culture		Subculture	
Fraction	Number of Generations	Fraction	Number of Generations
1B	4.0	1B	NG
1B	1.8	1B	NG
		WS	5.3
1B	3.1	1B	NG
		WS	NG
2A	3.5	2A	2.4
		WS	4.2
2A	4.3	2A	NG
		WS	4.7
2A	3.1	2A	NG
		WS	NG
1B + 2A	4.1	1B + 2A	NG
		WS	3.4
1B + 2A	3.5	1B + 2A	NG
		WS	--

Table 7 showed that medium containing fraction II erratically supported Giardia growth while medium containing fractions II + III consistently supported growth. Table 12 showed that fraction 3B enhanced the growth promoting activity of fraction 2A. For these two reasons, subculture attempts were made with M5 media that had the combinations of fractions 1B + 3B or 2A + 3B in place of whole serum. The protozoa grew just as well in subculture as they did in primary culture in both types of media. (Fig. 10).

For another type of subculture experiment Giardia trophozoites were grown in medium containing 1B, 2A or 1B + 2A in place of whole serum. These protozoa were subcultured into 3 different media: medium containing whole serum (control), medium containing the particular fraction and medium containing the particular fraction plus fraction 3B. The results of 5 experiments done this way are shown in Table 14. In each case the Giardia trophozoites were successfully subcultured in medium supplemented with 3B, but not in medium containing the particular 1B, 2A or 1B + 2A fraction without 3B.

4. Serial subcultures.

Table 15 shows the growth response, in generations per culture, for 2 subculture series of Giardia grown in medium with 1B + 3B substituted for whole serum. The number of generations increased in the first 3 cultures of series 1 from 2.7 to 5.0. This series was cut short by contamination. The number of generations also increased in the first 3 cultures of series 2 from 3.0 to 4.2. From this point on the growth response remained fairly constant until the eighth,

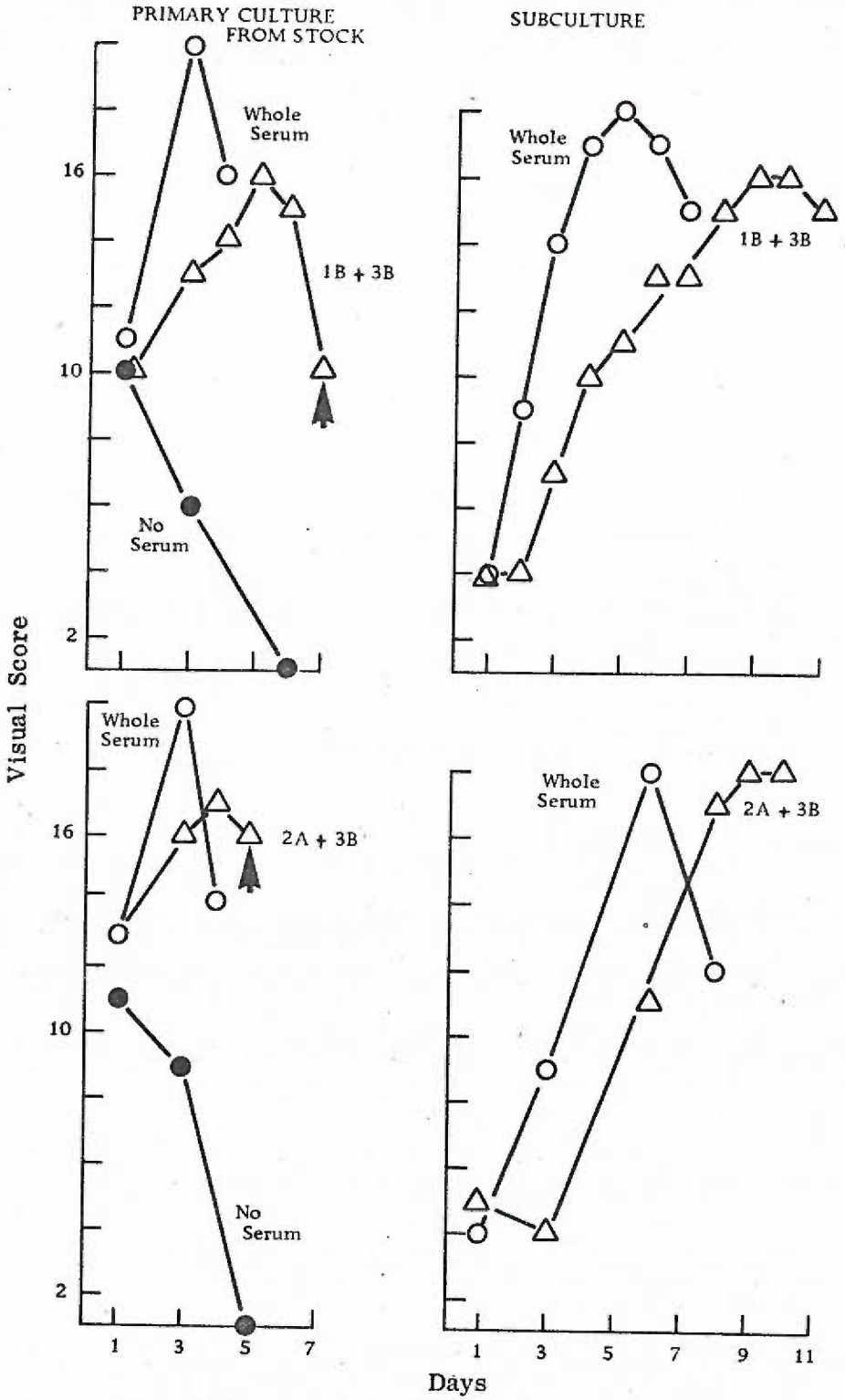


Fig. 10. Growth response in primary culture and subculture to G-200 Sephadex fractions 2A + 3B and 1B + 3B in M5 medium. Each point is the average of three replicate cultures. Standard deviations are always $\pm$ 1. All preparations were from serum pool 30. Arrows indicate source of inoculum for each of the subcultures.

Table 14. Growth response in primary culture and in subculture to G-200 Sephadex fractions in M5 medium. At peak of primary growth organisms were subcultured into fractions indicated. WS is whole serum control; NG is no growth. All fractions were prepared from serum pool 30.

Source of Inoculum for Subculture		Subculture	
Fraction	Number of Generations	Fraction	Number of Generations
1B	3.0	1B	NG
		1B + 3B	3.8
		WS	--
2A	2.6	2A	NG
		2A + 3B	4.5
		WS	4.5
2A	3.3	2A	NG
		2A + 3B	3.1
		WS	5.5
2A	2.1	2A	NG
		2A + 3B	3.2
		WS	--
1B + 2A	3.1	1B + 2A	NG
		1B + 2A + 3B	3.1
		WS	3.5

Table 15. Growth response in serial subculture to G-200 Sephadex fraction 1B + 3B in M5 medium. Series 1 was carried from 9/70 to 10/70; series 2 from 11/70 to 3/71. Method of calculating specific activity is in the appendix. All preparations were made from serum pool 30.

Culture	No. Generations		Specific Activity	
	Series 1	Series 2	Series 1	Series 2
1B+3B - 1st	2.7	3.0	600	885
2nd	3.7	3.1	825	800
3rd	5.0	4.2	1220	1080
4th	Contaminated	3.4		890
5th		2.8		705
6th		4.3		1050
7th		3.4		780
8th		1.6		410
Ave. ± S.D.	3.8± 0.7	3.2± 0.4	880± 180	815 ± 90

Average specific activity of 22 replicate primary cultures of whole serum pool #30 is 255 ± 20; average number of generations is 5.2 ± 0.2.

and last subculture attempted, which had a low growth response of 1.6 generations.

Table 16 shows data for a single serial subculture series in medium containing 1B+4x crystallized human albumin in place of whole serum. The albumin was obtained from Nutritional Biochemical Co. (Cleveland, Ohio). In this case the eighth and last subculture attempted gave a very typical growth response of 4.1 generations. The average number of generations in this series was 4.2 ± 0.3 and the average specific activity, in generations per concentration of serum protein (gm/ml) in the medium, was 1060 ± 90. The average number of generations in the two 1B + 3B series (Table 15) were 3.8 ± 0.7 and 3.2 ± 0.4, and the average specific activities were 880 ± 180 and 815 ± 90. For 22 experiments done with whole serum (pool 30) control medium the average number of generations per culture was 5.2 ± 0.2 and the average specific activity 255 ± 20.

So, for the 1B + 4x medium there was an average growth response of 80% of control ($4.2 \div 5.2$ generations), and more than a 4 fold increase in specific activity ($1060 \div 255$). For the 1B + 3B medium there was an average growth response of 68% of control ($\frac{3.8 + 3.2}{2} \div 5.2$ generations) and a more than 3 fold increase in specific activity ($\frac{815 + 880}{2} \div 255$).

Two separate experiments, one with 1B + 3B-grown cells (Fig. 11), and one with 1B + 4x-grown cells (Fig. 12) were set up according to the following protocol. Cells grown in their respective media were

Table 16. Growth response in serial subculture to G-200 Sephadex fraction 1B + commercial 4x crystallized albumen in M5 medium. Fraction 1B was made from serum pool 30; the albumen (lot #5865) was obtained from Nutritional Biochem. Co. (Cleveland, Ohio). Method of calculating specific activity is in the appendix.

Culture	Number of Generations	Specific Activity
1B+4x - 1st	3.0	750
2nd	3.1	780
3rd	4.7	1180
4th	4.5	1120
5th	6.1	1530
6th	3.9	980
7th	4.4	1100
8th	4.1	1030
Ave ± S.D.	4.2 ± 0.3	1060 ± 90

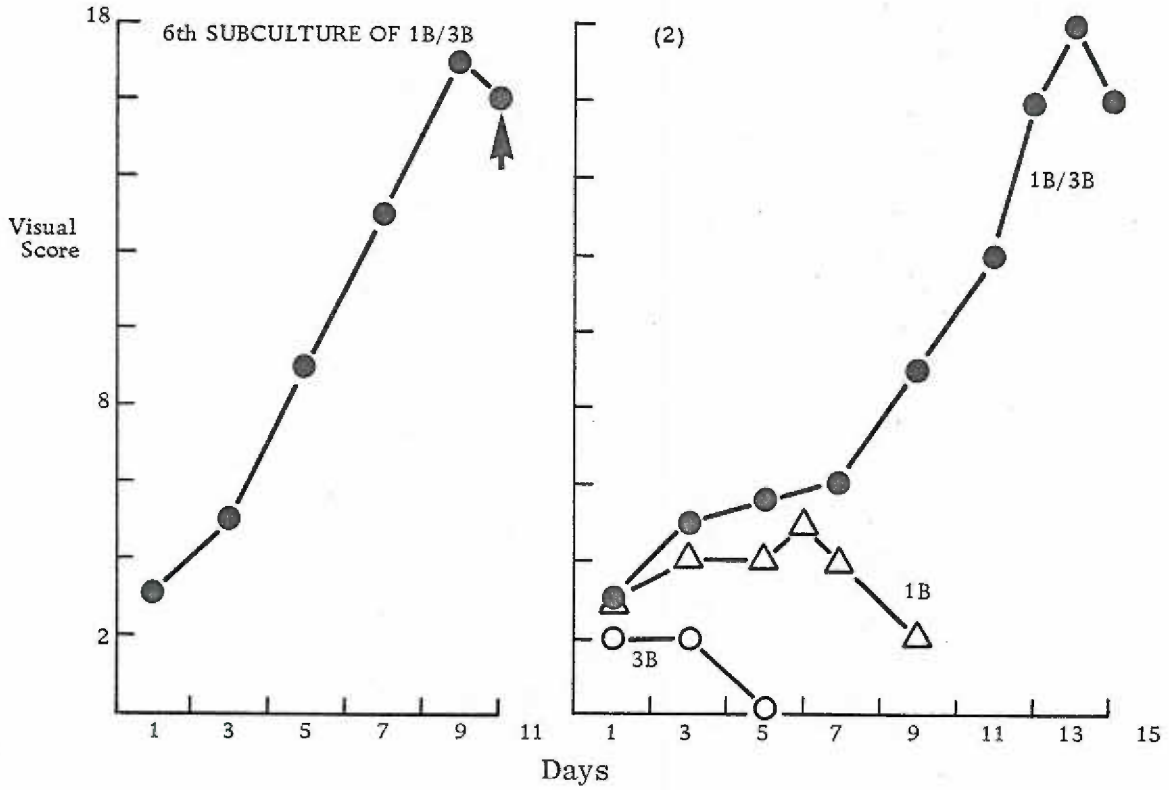


Fig. 11. Attempt to subculture organisms grown in medium containing 1B+ 3B into medium containing 1B or 3B. Each point is the average of two replicate cultures. All preparations were made from serum pool 30. Arrow denotes point at which 6th subculture of 1B+ 3B was subcultured into each of the variables in (2).

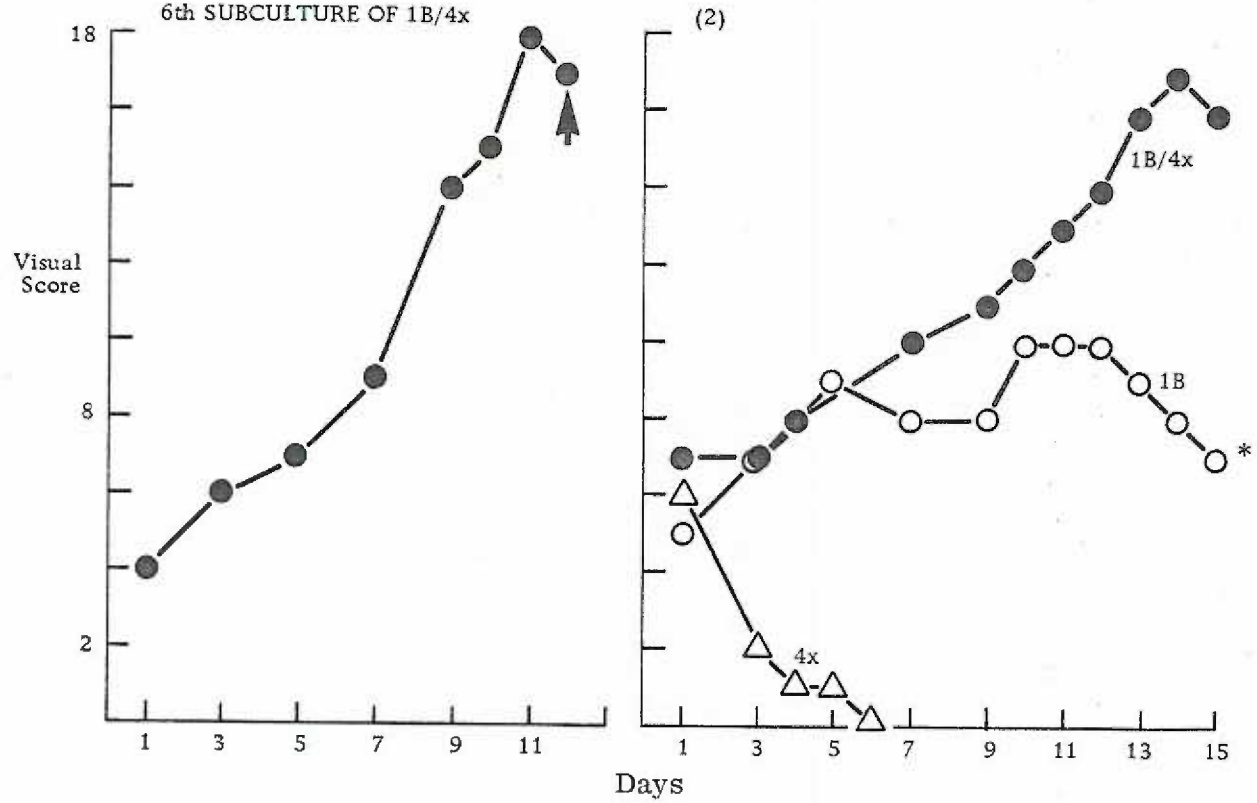


Fig. 12. Attempt to subculture organisms grown in medium containing 1B+4x into medium containing 1B or 4x. Each point is the average of two replicate cultures. Preparation 1B was made from serum pool 30; albumen (4x crystallized - #5865) was obtained from Nutritional Biochemical Co. Arrow denotes point at which 6th subculture was subcultured into each of the variables in (2). Asterisk denotes point at which organisms from the 1B culture in (2) were transferred into fresh 1B medium; these organisms failed to grow.

subcultured into three different media: M5 containing both the 1B and 3B or 4x component and M5 with only the 1B and only the 3B or 4x component in place of whole serum. The figures show that after 6 subcultures, Giardia still require both the high molecular weight component (1B) and the low molecular weight component (3B or 4x) to grow. Media containing either fraction 1B, 3B or 4 x albumin in place of whole serum did not support continued growth. This demonstrated that Giardia had not adapted to one or the other of the two serum fractions.

E. Attempts to Identify the High Molecular Weight Contribution to Giardia Growth.

1. Identities between the 1B and 2A fractions.

In Table 11 it was shown that in medium where serum was substituted with either fraction 1B or 2A primary growth measured as the number of generations was approximately 65% of whole serum control. Doubling the amount of 1B or 2A produced a growth response almost as good as combining the two fractions; this response was higher than the response to media containing 1B or 2A alone. This suggests that the growth promoting substance was the same in each of the 2 fractions and was split rather evenly between them. So, attempts were made to find substances common to fractions 1B and 2A.

The results of acrylamide gel discontinuous electrophoresis (by 2 different methods) on the 6 Sephadex serum fractions are depicted in Fig. 13. In method A the arrows denote 3 bands common to

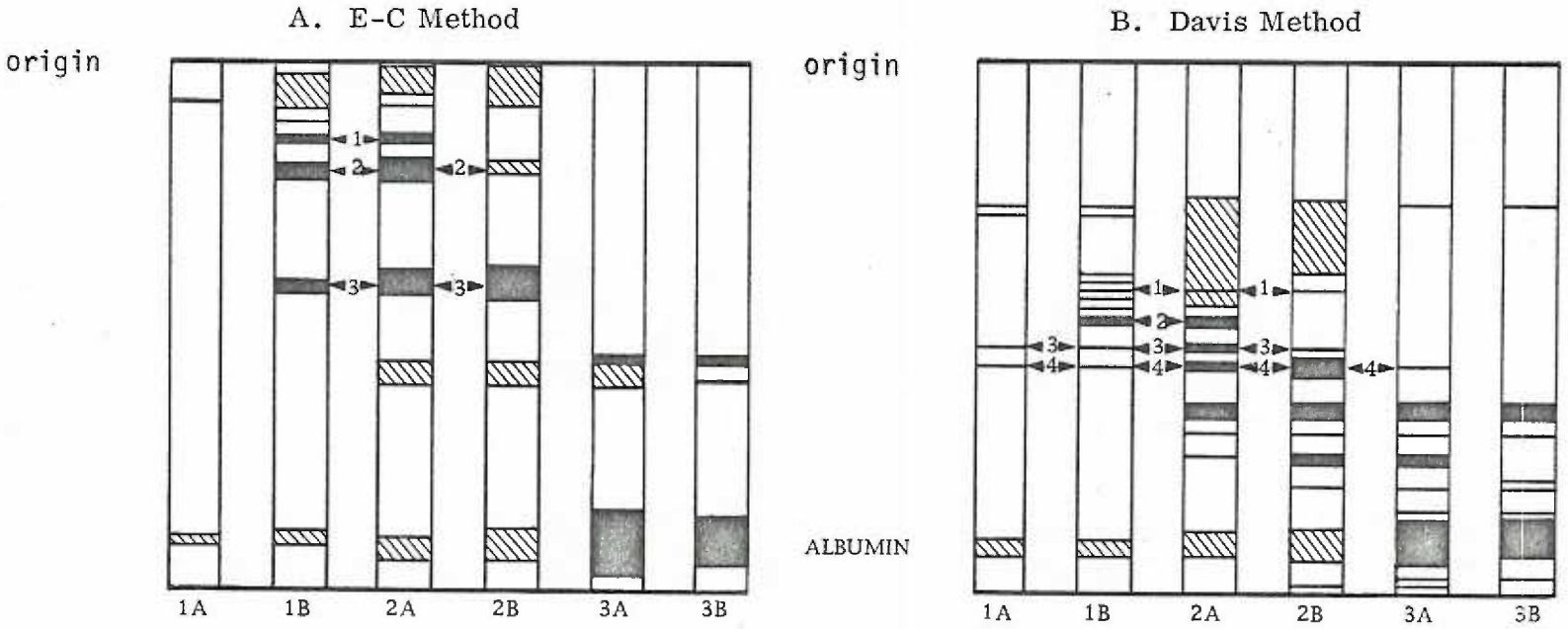


Fig. 13. Discontinuous gel electrophoresis of the 6 fractions from G-200 Sephadex gel filtration. A. E-C method is described in the methods section. The stained gel slab was observed over a lighted box and the original figure drawn by Dr. Meyer and myself. B. Davis method is described in the methods section. The stained gel cylinders were observed in Dr. Dennis Burger's laboratory and the original figure drawn there. Arrows indicate bands common to fractions 1B and 2A. Bands that are drawn with slanted lines indicate diffuse staining in the original gels. All Sephadex fractions were prepared from serum pool 30.

1B and 2A plus traces of albumin in each. The band denoted by arrow 1 is present only in 1B and 2A. In method B there are 4 bands and traces of albumin common to both 1B and 2A. Only band 2 is present in just 1B and 2A.

Immunoelectrophoresis of the 6 fractions against rabbit anti whole human serum (prepared by Dr. Dennis Burger, Division of Surgery, Veterans Hospital, Portland, Oregon) showed only two precipitation lines common to 1B and 2A. The concentration of protein in each of the 6 fractions was set at 1 to 2 mgm/ml.

Ouchterlony double diffusion was selected to test for the presence of specific human serum antigens in the 6 fractions. The specific antiserum was pipetted into the center well and the fractions were pipetted into the 6 wells surrounding the center. The concentrations of the fractions used were equivalent to what they would be in whole serum (see Table 10). Table 17 shows the results of experiments done with 10 different antisera. Present in both 1B and 2A were IgA, IgG and haptoglobin.

Quantitation of the 3 antigens common to 1B and 2A was done by serial two-fold dilution of each of the fractions in which they were present. The specific antiserum was pipetted into the center well, and the dilutions of the particular fraction pipetted into the surrounding wells. The data in Table 18 approximate the quantity of each of the 3 antigens in each of the fractions tested. Haptoglobin, which is present in 3 fractions, is distributed in equal amounts in fractions 1B and 2A. The amount in either of those fractions is 4 times greater than the amount in 2B (maximum dilution of 16 vs

Table 17. Double diffusion data for 6 G-200 Sephadex fractions made from serum pool 30. All fractions were used at concentrations equivalent to what they would be in whole serum (Methods Section II-C-5). Listed under each fraction are only those specific antisera that gave reactions with that fraction. Gels were observed over reflected light. All 10 antisera were tested against each fraction.

Fraction					
1A	1B	2A	2B	3A	3B
IgA	IgA	IgA			
IgG	IgG	IgG	IgG	IgG	
	Haptoglobin	Haptoglobin	Haptoglobin		
α 2 Macro-globulin	α 2 Macro-globulin	Cerulo-plasmin	Cerulo-plasmin	Transferrin	Transferrin
β Lipoprotein	β Lipoprotein		Transferrin	Hemopexin	Hemopexin
G-C Globulin	Anti-Trypsin		Hemopexin	G-C Globulin	G-C Globulin
	G-C Globulin			Anti-Trypsin	Anti-Trypsin

Table 18. Relative quantitation of those antigenic substances present in both 1B and 2A G-200 Sephadex fractions. Serial twofold dilution was done with each fraction. The number indicates the highest dilution at which a precipitation line still occurred. Gels were observed unstained over reflected light.

Specific Substance Tested For	Fraction				
	1A	1B	2A	2B	3A
IgA	2	16	32	--	--
IgG	8	16	128	128	4
Haptoglobin	--	16	16	4	--

max. dil. of 4). The amounts of IgA in fractions 1B and 2A are within one dilution of each other and far exceed the amount in fraction 1A (16 and 32 vs 2). IgG is present in greatest amounts in fractions 2A and 2B where it occurs to the same titer. The amount of IgG in either of those fractions is 8 times greater than that in fraction 1B, 16 times that in 1A and 32 times that in 3A.

2. Experiments with unusual sera.

When serum heated at 65 C for 2 hours was tested earlier (see Results section A) there was no loss in growth promoting ability. The serum underwent noticeable changes, however. It turned pea soup green and passed through a Millipore filter more rapidly than untreated serum. Investigating this further by Ouchterlony double diffusion methods, it was found that anti-haptoglobin antiserum no longer gave a precipitation line with the heated serum. Fig. 14 A and B show the growth responses to 2 different preparations of serum heated at 65 C for 2 hours. Giardia grew slightly better in it than in unheated serum. Specific antisera against IgG and IgA gave precipitation lines when tested with the heated serum by the Ouchterlony method. Unheated (control) serum gave precipitation lines with antisera against IgG, IgA and haptoglobin.

Fig. 14 E shows the growth response to medium containing serum from a patient with Bruton's disease. This serum had a lower total protein concentration than normal serum. Consequently, a higher volume of Bruton's serum was used in experimentation to compensate for this difference. Radial diffusion evidence showed no IgA antigen (less than 0.2 mgm/ml) and a depressed amount of IgG (approx. 1 mgm/ml).

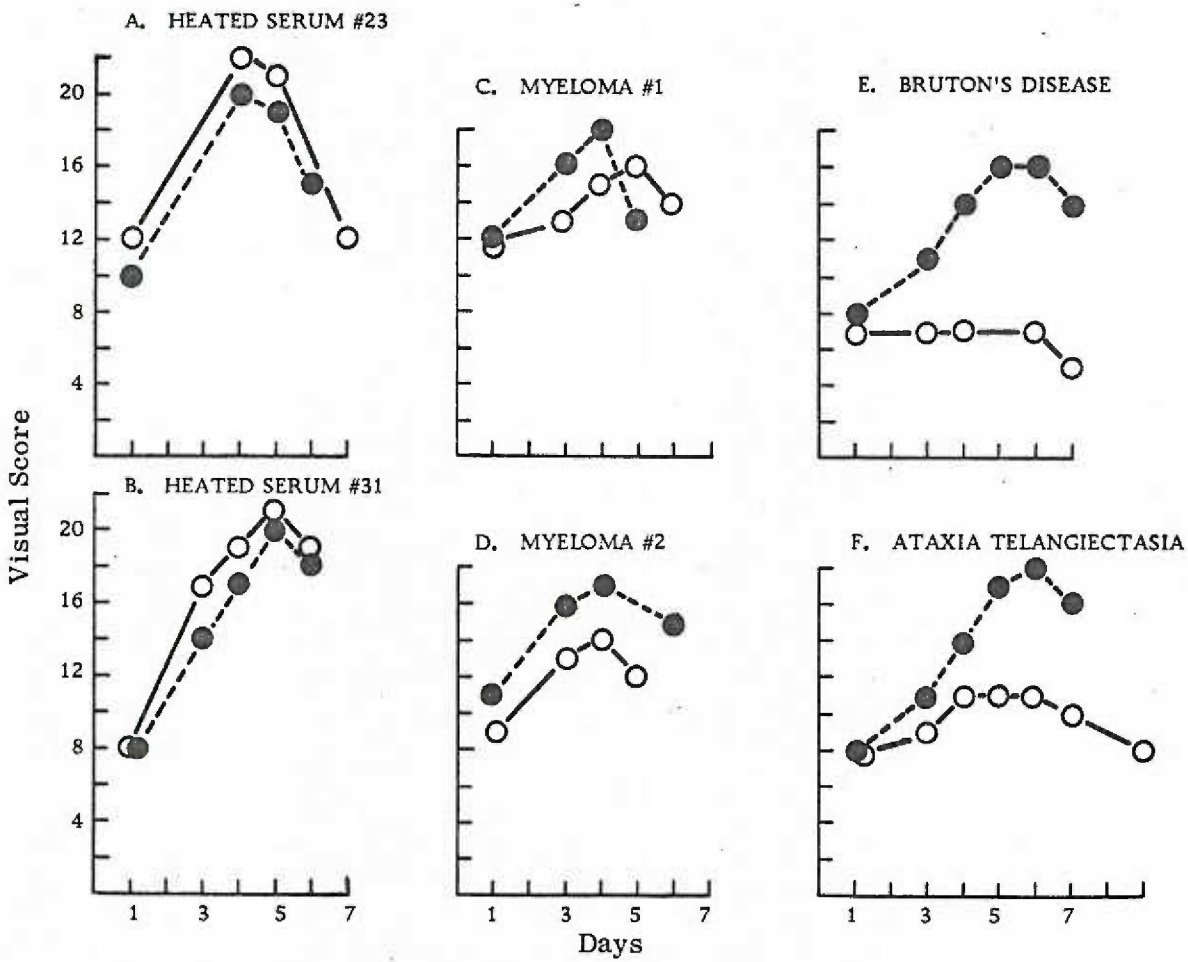


Fig. 14. Growth response to different types of sera in M5 medium compared to response to normal or untreated serum. Each point is the average of three replicate cultures; all points have a standard deviation of ±1. Dotted line is normal serum control in all 6 graphs. A and B--Two different normal sera heated in a water bath at 65 C for 2 hours. C and D--Serum from two people with IgA myelomas. E and F--Serum from a person with Bruton's disease, and a person with Ataxia telangiectasia; both of these sera have no IgA.

Normal serum concentration of IgA is 1.5 to 4 mgm/ml (90) and IgG 12 to 18 mgm/ml (91). Ouchterlony double diffusion tests showed haptoglobin to be present. This serum did not have growth promoting activity.

Fig. 14 F shows the Giardia growth response to medium containing serum from a patient with ataxia telangiectasia. This serum had a normal IgG content (12.8 mgm/ml), but no IgA as determined by radial diffusion. This serum supported only minimal growth.

Fig. 14 C and D show experiments where sera from 2 different people with IgA multiple myelomas were substituted for normal serum in M5 medium. The protein concentrations of these two sera were approximately equal to the concentrations of normal serum controls. The IgG content of # 1 was 11.3 mgm/ml and # 2 9 mgm/ml; both values were determined by radial diffusion. There were, of course, unusually high amounts of IgA in each of the two sera. Radial diffusion readings for IgA in serum # 1 were off scale (greater than 5 mgm/ml); and paper electrophoresis showed an unusually high spike for IgA in serum # 2.

3. Experiments with normal human serum.

A sample of M5 medium in which Giardia had grown from lag to stationary phase was freed of Giardia by centrifugation. This spent medium was serially two-fold diluted and the dilutions tested for reaction with specific antisera in Ouchterlony gel slides. The antigens tested for were IgA, IgG, haptoglobin, albumin and transferrin.

A sample of the original sterile M5 medium was tested for antigens in the same manner. In 2 separate experiments there was no difference between the spent and control media in the maximum dilution which gave precipitation with any of the 5 antisera. These crude estimates demonstrated that none of the 5 serum antigens was detectably depleted from medium in which Giardia had grown.

A thick suspension of Giardia trophozoites, which had been harvested from stock cultures, was sonicated extensively (3 minutes at a setting of 50 on a Bronwill Biosonik, Bronwill Co., Rochester, N. Y.). The clear sonicate was pipetted into the center well of an Ouchterlony gel slide. Into the 6 surrounding wells, anti IgA, whole serum, anti IgG, whole serum, anti albumin and whole serum were pipetted in the order presented. The experiment was designed to test for the presence of any of the 3 antigens inside Giardia and for any non-specific anti-Giardia activity in normal sera. Further, if there was anti-Giardia activity this experimental setup would show if that activity was in the IgA or IgG portion of normal serum. The results, however, were negative. Precipitation lines occurred only between whole serum and each of the specific antisera. The experiment was repeated with the same results.

F. Change in Giardia Growth Response

Table 19 shows the growth response of Giardia in primary culture in the same medium during 2 different time periods. During the 6/70 to 11/70 period the growth response in medium containing 1B in place of whole

Table 19. Primary growth response of serial stock culture Giardia. All G-200 Sephadex preparations were made from serum pool 30. Growth response to whole serum controls was the same from 6/70 through 3/71. Growth is expressed as % of control activity.

Date	G-200 Fraction			
	1B	2A	1B + 2A	1B and/or 2A plus 3B
6/70 to 11/70	63 ± 7 (8 replicates)	64 ± 4 (16 replicates)	89 ± 4 (5 replicates)	78 ± 5 (4 replicates)
1/71	33 43	23 14	--	--
2/71	38	--	39	17
3/71	--	--	--	11 0

* The average number of generations in M5 medium with whole serum during the period 6/70 to 3/71 was 5.2 ± 0.3. There was no decrease in growth response observed during this entire period.

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F. Change in Giardia Growth Response

Table 19 shows the growth response of Giardia in primary culture in the same medium during 2 different time periods. During the 6/70 to 11/70 period the growth response in medium containing 1B in place of whole

serum was 63 ± 7% of control, but during 1/71 and 2/71 the growth response in medium containing the same 1B preparation was only 38 ± 3%. Giardia growth response to media containing other G-200 Sephadex serum fractions (Table 19) also dropped during the 1/71 to 3/71 period. However, during the 2 time periods there was no difference in growth response of the protozoan in medium containing whole serum from pool 30 (see footnote Table 19).

There were 2 possible explanations for this changed growth response: one was that the strain of Giardia (designated 64R), which was used in all the experiments described here and which was kept in serial culture, underwent a change; the other was that the serum fractions, which had been prepared from serum pool 30, had lost some activity during storage at -20 C.

In October of 1970, 2 different serial subcultures were initiated from strain 64R. One was established in medium containing 1B + 3B in place of whole serum, the other in medium containing 1B + 4x crystallized human albumin. Each of these was carried through 8 serial subcultures from 11/70 to 3/71 (see Tables 15 and 16) -- the same time period that the primary Giardia (strain 64R) growth response to these same 1B and 3B preparations diminished. This indicated that the ability of these preparations to support Giardia growth had not changed, and that the 64R strain had indeed changed in serial culture.

In late 1968 and early 1969 several preparations of the 64R strain were frozen in glycerol and stored at -70 C according to the method of Meyer and Chadd (92). A few months after that, samples of these preparations were thawed and successfully established in stock culture. This

work was done by Judy Chreist and E. A. Meyer (unpublished data).

After the change in Giardia growth response was observed attempts were made to initiate cultures of these same frozen 64R strain organisms. In 16 attempts, now 2 years after freezing, 10 cultures did not grow, 3 were grossly contaminated and 3 did grow. However, the 3 cultures that grew had in common a low level bacterial contaminant which was gram +, coccoid and anaerobic. No further attempts were made to initiate stock cultures from the frozen 64R strain preparations.

DISCUSSION

A. *Giardia* Culture System

The 1970 paper by Meyer (11) is the only report in the literature of the axenic cultivation of *Giardia*. There are, however, several reports of monoxenic culture of the protozoan; these are discussed below.

Karapetyan has reported culturing *Giardia* trophozoites from man (9); the culture was initiated in the presence of chicken fibroblasts and *Candida gulliermondii*, but for continued growth of the flagellate only *Candida* had to remain in the system. In a later report on this same system (93), Karapetyan measured the *Giardia* generation time to be 36 to 38 hours and the increase in cell number to be 10 to 14 fold (3 to 4 doublings).

Karapetyan also successfully cultured rabbit *Giardia* along with *Saccharomyces cerevisiae* (10). He did not report generation time or increase in cell number in this case, but did state that the peak concentration in his cultures was 3 to 5 x 10^5 protozoa per ml. Roux and Ecalle (94), studying *Giardia* from the rabbit in culture with *S. cerevisiae*, reported generation times of 30 to 40 hours and 10 to 20 fold increases in trophozoite number (3+ to 4+ doublings). Ecalle (95), studying these same organisms in the same basal medium supplemented with pancreatic juice noted similar increases in the number of *Giardia*, but a shorter generation time of 20 to 30 hours.

In the present work in which rabbit *Giardia* have been cultured in the absence of other organisms in M5 medium containing whole serum, the observed generation time was 18.1 $\pm$ 1.6 hours, the number of doublings

5.5 $\pm$ 0.3, and the peak concentration 1 to 3 x 10^5 protozoa per ml. These data are not directly comparable to the data collected by earlier workers (9, 10, 93, 94, 95) since they used mixed cultures of Giardia and yeast, medium different from M5 and, in one case (9, 93), Giardia from man rather than the rabbit. The data obtained in the present study, however, do indicate that Giardia grow vigorously and to relatively high concentration in this in vitro system.

The visual scoring method developed in this work was shown to be a reasonably accurate way of determining Giardia trophozoite concentration during logarithmic growth phase, although somewhat less accurate than electronic cell counting. The problem with the electronic counting method was that the measurement of the organisms in a culture tube required treatment such that the culture was no longer usable. The visual scoring method permitted organisms in the same culture tubes to be counted repeatedly during an experiment which greatly reduced the number of culture tubes necessary for a particular experimental variable. This reduction allowed for the study of a greater number of variables within a given experiment than would otherwise have been possible, and for a saving of materials - particularly prepared serum fractions.

B. Serum Fractionation Studies

The first serum fractionation experiment showed that all of the Giardia growth promoting substance was non-dialyzable. This was necessary information for fractionation experiments to follow, as all serum fractions were equilibrated by dialysis with physiological Tris-buffered saline (bs)

before incorporation into test media. Since the dialysis membrane allows only crystalloids or the relatively small molecules of serum to pass through it (96), this experiment demonstrated that the colloidal macromolecules of serum (viz. the proteins), and/or substances closely associated with serum macromolecules, were responsible for promoting Giardia growth.

The 50% ammonium sulfate precipitate of serum also retained all of the growth promoting activity. However, of the 2 fractions created by 20% of saturation ammonium sulfate treatment of the 50% precipitate, only the 0-20% fraction (20P) had growth promoting activity when substituted for whole serum in M5 medium. G-200 Sephadex ultrafiltration of these 2 fractions (Fig. 4) showed that there was no 7S peak material in the 20-50% fraction (20Su). This implicated 7S peak material, which is the molecular weight range of 100,000 to 200,000 (97), as at least part of the serum contribution to Giardia growth. The 20P fraction had only a small amount of 4S peak material and the 20Su fraction a large amount. The fact that the 20Su fraction enhanced the growth response to the 20P fraction when combined with it, suggested that 4S peak material was also contributing to Giardia growth. Material in the 4S peak is of lower molecular weight than 100,000 (97).

These fractions were not subjected to further ammonium sulfate precipitation as there was already a loss of growth promoting activity as a result of the 50-20% double precipitation. Instead, whole serum was studied by ultrafiltration through a membrane which had the specifications of allowing the passage of molecules of less than 100,000 molecular weight.

The purpose of this procedure was to separate the 4S peak material from the rest of serum. The G-200 Sephadex tracings of the 2 fractions created by this procedure (Fig. 5) showed that 19S, 7S and 4S peak material was present in each fraction. Since a qualitative separation was not achieved, the 2 fractions were not further studied and the membrane was not used further to separate growth components in serum.

G-200 Sephadex preparative treatment of the 50% ammonium sulfate precipitate produced the fractions designated I (19S peak), II (7S peak) and III (4S peak). The growth response of Giardia in medium containing fraction II in place of whole serum was erratic, while the response to medium containing II + III was consistently good. Fraction I was not essential as demonstrated by the consistent growth response and the successful subculture of Giardia in medium without it. However, in some cases there was a small growth response of the protozoan in medium containing only fraction I in place of whole serum. There was also a slightly better growth response in medium containing I + II + III than in medium containing II + III. One explanation for this observation is that the growth promoting substance in fraction II was also present in fraction I.

Attempts to study fraction II by DEAE cellulose treatment and fractions II and III by G-100 Sephadex column filtration were non-productive. DEAE cellulose treatment destroyed the growth promoting activity in fraction II. G-100 Sephadex treatment excluded all of fraction II and gave a single unimodal curve of fraction III (Fig. 8). Since the G-100 Sephadex procedure gave no advantage in separation over G-200 Sephadex it was not pursued.

Each of the 3 G-200 Sephadex fractions was now split in half. Of the 6 fractions tested singly in substitution for whole serum in M5 medium, either 1B or 2A supported Giardia growth to approximately 65% of the whole serum control. Combining the 2 fractions resulted in a growth response of 89% of control. This was considered to be a good growth response as there was a 10% loss of protein in the G-200 Sephadex filtration procedure (Table 10).

The reconstituted preparation of all 6 serum fractions yielded a growth response (65%) lower than that obtained (89%) in medium in which the only serum constituents were 1B and 2A. This suggested that something in the other 4 fractions was growth inhibiting. Fraction 3B was eliminated as the source of this inhibitory activity since medium containing 2A + 3B gave better Giardia growth than medium containing 2A without 3B.

In fact, fraction 3B proved to be necessary for continued growth of the flagellate, since primary cultures of Giardia in medium containing 1B, 2A or 1B + 2A could not be subcultured unless 3B was present in the subculture medium. One explanation for this was that stock Giardia had pooled enough material of the 3B type to undergo several divisions during primary culture. When the pool was used up, however, the cells could no longer grow (in subculture) unless more 3B material was available.

To show that Giardia could be continuously cultured in medium containing 1B + 3B substituted for whole serum, serial subcultures were initiated. The first series was carried successfully through 3 cultures before it was contaminated with bacteria. The second series was carried successfully through 8 cultures before it was terminated. In the last

culture of this series, the growth response dropped to 1.6 generations of Giardia. Since the protozoa in the preceding culture underwent 3.4 generations, this represents a difference of 1.8 generations. There is no ready explanation for this decreased growth response. It should be pointed out, however, that it is not out of the range of observed random differences from culture to culture. In Table 15 it can be seen that there was a total of 26 generations of Giardia through 8 serial cultures in this serum-substituted medium.

Giardia were also successfully serially subcultured in medium containing 1B + commercial 4x crystallized human serum albumin. In this single series of 8 cultures there was a total of 33.5 generations of Giardia and the growth response of the last culture was no different from (that of) the others. The average number of protozoan generations per culture (4.2) was approximately 80% of that in primary cultures in medium containing whole serum. The generation time, 30 hours, was longer than the 18 hour generation time observed for whole serum primary cultures. There was, however, a 4 fold increase in specific activity of the serum substitutes over whole serum.

In order to show that the Giardia had not adapted to one or the other of the 2 components of 1B+3B or 1B+4x substituted medium, subcultures were attempted into medium containing 1B and 3B, respectively (from cells grown in medium containing 1B + 3B) and 1B and 4x, respectively (from cells grown in medium containing 1B + 4x). None of the media supported Giardia growth.

Subculture experiments have demonstrated that Giardia strain 64R from the rabbit can grow continually in medium whose serum has been substituted with 2 serum fractions, and that both of these fractions are necessary for continued Giardia growth. The total protein of these 2 fractions represents 20% of the total protein in whole serum. One fraction is in the molecular weight range of 200,000 by virtue of its location between the G-200 Sephadex 7S and 19S peaks, and the other is of lower molecular weight.

C. Nature of the Low Molecular Weight Serum Component Contributing to Giardia Growth

In growth studies with fractions prepared from the 3 whole G-200 Sephadex peaks, the supernatant S from 50% of saturation ammonium sulfate precipitation could successfully replace fraction III in combination with fraction II. In growth studies with fractions prepared from the 6 split G-200 Sephadex peaks, 4x albumin could successfully replace fraction 3B in combination with fraction 1B. So, components III, S, 3B and 4x albumin all represent interchangeable and essential contributions to the growth of Giardia, and all contain albumin. However, at least 3 of the 4 fractions also contain other materials.

Schultze and Heremans (97) have demonstrated that the 4S peak from G-200 Sephadex gel filtration of whole serum (fraction III) contains 13 other substances besides albumin, and that the lower molecular weight portion of that peak (roughly equivalent to fraction 3B) contains 9 other substances. In the most definitive disc electrophoretic analysis (Fig. 13 b) we detected 7 other bands in 3B besides the predominant albumin band. The S fraction from a single 50% ammonium sulfate precipitation certainly

contains other serum substances, although it too is primarily albumin (98). The commercial preparation of albumin which had been recrystallized 4 times according to its specifications, may also have had contaminating substances.

Dixon and Maurer (99) reported that their commercial preparation of bovine serum albumin (BSA) contained a small percentage of bovine gamma globulin. Linscott (100) reported that 7 commercial preparations of rabbit serum albumin were all contaminated with BSA. In neither of these studies were any other contaminating substances tested for. The 4x crystallized human serum albumin (HSA) preparation used in this study was examined for contamination by other human serum components. A 10 mgm/ml HSA solution was tested for the presence of the following antigens: IgG, IgA, haptoglobin, transferrin, hemopexin, ceruloplasmin, β lipoprotein, α-2 macroglobulin, α-1 antitrypsin and GC globulin. None of these antigens was detected. The possibility of yet other substances being present in the commerical HSA preparation is still possible, since there are many other human and other animal serum constituents we did not test for. Nevertheless, the results suggest that albumin is the low molecular weight contribution of human serum to the growth of the 64R strain of Giardia from the rabbit.

Albumin has been identified as part or all of the serum contribution to the growth of tissue culture cells (42, 49, 50, 51, 101), pathogenic bacteria (21, 53), a Mycoplasma (63), a rickettsia (52) and 2 species of the flagellated parasitic protozoan Trichomonas (54). Later studies of some of these systems have shown that albumin (or substances closely associated with it) may have any of several different functions.

Ham (66) showed that linoleic acid, which is closely associated with HSA, was the actual growth promoting substance for Chinese hamster cells. In the case of Leptospira pomona (65) BSA that had been stripped of fatty acids by extraction was itself necessary for growth along with oleic acid. Neither substance alone supported growth. Since the stripped albumin could be replaced with starch it was considered to have an adsorbing function in the growth medium. Oleic acid in the absence of albumin was toxic to the leptospires. The albumin protected the cells by adsorbing the oleic acid, and fed them by slowly releasing it. In the case of Bordetella pertussis (53) albumin serves purely as a detoxifying adsorbing agent for the fatty acids generated by the bacterium in culture. Tritsch et al. (102) showed that hamster bowel carcinoma cells in culture could take up and degrade HSA, and Reyser (103) demonstrated that Sarcoma 180 cells in culture could do the same. In both cases labeled amino acids initially in the albumin were incorporated into cellular protein. However, both were short term studies done in media that did not support continued cell growth.

These examples from the literature illustrate 3 functions of albumin: a source of fatty acids, a detoxifying or adsorbing agent and a source of amino acids. The last function is unlikely in the case of Giardia since no depletion of albumin was detected from medium in which the flagellate had grown and no albumin was detected in sonicates of the protozoan. In both of these experiments albumin was tested for in Ouchterlony gels with anti albumin antiserum. The depletion experiment measurements were relatively crude as they were based on serial two-fold

dilutions of medium. It is possible that a small amount of albumin could have been depleted from the medium and gone undetected. It is also possible that any albumin taken up by Giardia was degraded rapidly, thereby leaving no detectable albumin antigen in sonicates of the protozoan.

With respect to the possibility that albumin may be a source of fatty acids for Giardia, Chen (104) has shown that there are 1.8 to 2.5 moles of fatty acid associated with each mole of the same brand of commercial HSA used in this study. Goodman (105) measured 1.8 moles of fatty acid per mole of albumin in his HSA preparation. So, fatty acids are commonly associated with HSA and might well be available for Giardia in the growth medium.

It remains for further study to determine the role that albumin plays in support of Giardia growth.

D. Nature of the High Molecular Weight Serum Component Contributing to Giardia Growth

Primary cultures of Giardia strain 64R grew equally well in medium containing 1B or 2A substituted for whole serum. Even better growth was observed in medium containing 1B + 2A, 2x 1B or 2x 2A. These facts suggested that a common growth promoting substance was distributed between fractions 1B and 2A. Consequently, a search was made for substances common to both fractions.

One disc electrophoresis method showed 3, and the other 4, common bands in 1B and 2A. The identity of 3 of these components, haptoglobin,

IgA and IgG was detected by Ouchterlony gel diffusion of 10 specific antisera against the 6 G-200 Sephadex fractions. The relative amounts of each of the 3 common antigens in any of the fractions in which they were present was also determined by Ouchterlony tests. Both IgA and haptoglobin were present in approximately equal concentration in 1B and 2A. There was a small amount of IgA in fraction 1A and a small amount of haptoglobin in fraction 2B. The quantity of IgG, on the other hand, was the same in 2A and 2B, but much lower in 1B.

Serum heated to 65 C for 2 hours in a water bath gave a slightly better growth response than normal serum when incorporated into the growth medium. Examination of this heated serum by Ouchterlony methods showed that there was no longer a reaction with anti-haptoglobin antiserum while anti IgA and anti IgG still gave precipitation lines. This meant that reactive haptoglobin antigen was no longer present in the heated serum. Heating serum apparently alters the antigenic structure of haptoglobin but not IgA or IgG - and heated serum is at least as good as, if not better than, unheated serum at supporting Giardia growth.

A variety of sera with abnormal IgA levels, obtained from people with various diseases, were tested for their ability to support Giardia growth. Serum from a person with Bruton's disease did not support Giardia growth when incorporated into M5 medium in place of normal serum. Radial diffusion tests of this serum showed IgA to be absent and IgG present at very low level; Ouchterlony tests showed haptoglobin to be present.

Serum from a patient with ataxia telangiectasia gave only a very slight growth response. This serum had no IgA and only a slightly depressed

amount of IgG compared to normal serum, as determined by radial diffusion.

Two sera from people with IgA myelomas gave good Giardia growth responses, although not quite as good as normal serum. These sera were rich in IgA and had slightly depressed levels of IgG.

The data from these experiments with abnormal sera are further evidence to implicate IgA as the growth promoting substance in fractions 1B and 2A - the high molecular weight contribution of serum to Giardia.

In studies of medium in which Giardia had grown no depletion of IgA, IgG or haptoglobin was detected; and none of those 3 serum components was detected in sonicates of Giardia. Both of these studies were done by Ouchterlony technique. This suggests that if the high molecular weight component is being taken up at all it is only in small amounts; furthermore that if any high molecular weight component is being taken up it is rapidly rendered non-antigenic.

In support of the argument that Giardia may utilize small amounts of IgA is information concerning the nature of IgA in human serum and in extravascular fluids. Ninety percent of the IgA in serum is of the 7S variety, the other 10% ranging from 9S up to 17S (106). In the effluent from a G-200 Sephadex column filtration of serum, one would expect to find most of the 7S IgA in the second peak which also contains most of the 7S IgG (see Table 18 and reference 97). The larger IgA polymers would be found in the first peak which also contains 19S IgM (97). The high molecular weight Giardia growth promoting substance was distributed equally between the second half of the 19S peak (fraction 1B) and the first half of the 7S peak (fraction 2A). Since the 7S IgA would primarily be in the 7S peak and to much lower concentration in the 19S peak, this

would suggest that the significant IgA was a polymer larger than 7S. In this case, the amount of the particular polymer of IgA which Giardia may utilize would be less than 10% of serum. A depletion of 9S, 11S or 13S IgA from serum which contains 90% 7S IgA would not be detected by the quantitative Ouchterlony technique used in this study.

With respect to the occurrence of IgG and IgA in the natural habitat of Giardia, Plaut and Keonil (107) reported that there was 4 to 5 times as much IgA as IgG in samples of fluid from the human small intestine, and Crabbe' and Heremans (108) have shown that 80% of the antibody cells lining the human small intestine produce IgA. This means that more IgA than IgG probably is available in the natural environment of the parasite, while the reverse is true in serum (91).

In his review of IgA literature, Tomasi (109) also noted that IgA is the predominant class of immunoglobulin in small intestinal fluid as well as in other human extravascular fluids including saliva, colostrum, urine and tears. He states that the predominant class of IgA in these fluids is 11S in size. The predominance of the 11S class of IgA in extravascular fluids is also reported in a review by Collins-Williams et al. (110). In both papers the 11S molecule is described as consisting of a dimer of two 7S IgA molecules plus a "transport piece" of smaller size. This transport piece is not present in serum IgA (109, 110). However, the fact that IgA is in dimer form in the intestine means that IgA available to Giardia in vivo is of greater molecular size than 7S.

It is of note here that albumin is also present in the small intestine (15), and that both albumin and IgA are present in bile (111). So, both

of the postulated Giardia growth-promoting substances present in serum are also readily available in the natural environment of the parasite.

E. Change in Giardia Growth Response

For a period of 6 months during this research, stock cultures of Giardia in medium containing whole serum grew quite well on initial transfers into medium containing fractions 1B and/or 2A, or fraction 1B or 2A plus 3B in place of serum. However, in an ensuing 3 month period stock-cultured organisms inoculated into medium containing the same fractions grew poorly; subsequent subcultures failed to grow at all. That this was a change in serially stock-cultured Giardia and not a reduced ability of the serum fractions to support growth, was demonstrated by the successful serial propagation of other cultures of the protozoan in medium containing 1B + 3B or 1B + 4x albumin; both series were initiated in the latter part of the 6 month period and carried through the subsequent 3 month period. It is important to note that during the entire 3 month period the stock-cultured Giardia grew quite well in medium containing whole serum.

Change in protozoan parasites during prolonged in vitro cultivation is well-documented in the literature. When Trichomonas vaginalis and T. gallinae are cultured in vitro they lose their virulence for test animals in a matter of a few months (112, 113). Stabler et al. (114) reported that for T. gallinae this loss of virulence could be reversed by serial animal passage of the parasite. However, they also reported that if the parasite was serially cultured in vitro for a long enough period of time (2 years), virulence could not be restored by animal

passage; in fact the trichomonads failed to grow at all on subsequent animal passage. In this case the parasite not only lost its virulence, but even its ability to survive in its natural host. The pigeons used as test animals in these studies had no immunity to trichomonads.

Honigberg et al. (115) reported that T. gallinae when passed in laboratory mice for 1 year (approximately 40 mice) increased its virulence for mice, but lost its ability to cause disease in pigeons. This mouse-passed organism grew in pigeons, but even after 5 serial bird to bird passes it was still avirulent in these animals. The original virulent strain, frozen in liquid N_2 for the one year period, was just as virulent when thawed as the year before, killing test pigeons in 6 or 7 days.

Histomonas meleagridis lost its ability to cause disease in turkeys after culture in vitro for a period of 9 weeks (116). Virulence could be restored at this time by bird to bird passage. However, after 4 months in culture the parasite would not cause disease even after bird to bird passage.

Vincent and Neal (117) showed that Entameba histolytica, originally isolated from a hamster, could cause disease in rats at any time during a 6 year period of culture in vitro. However, after the 6 years, continued in vitro culture resulted in a loss of virulence for rats during an additional 7 month period. Virulence for rats could be restored by passage in hamsters. The transfers were done by direct inoculation of the amebas into the liver of the hamster.

Trager (118) has reported that certain trypanosomes of the brucei subgroup undergo a morphological transformation when first cultured in vitro from their mammalian host. This also occurs when the parasite first

gets into the midgut of its insect intermediate host. However, when the parasites are artificially passed in laboratory animals in the absence of insect intermediates, they eventually lose their ability to transform and can no longer establish themselves in culture or in insect intermediate hosts.

One explanation for the observed change in growth response of Giardia is that the protozoan became more dependent on components in the whole serum in M5 medium. The parasite may have responded to its richer than necessary stock culture environment by losing certain capabilities, specifically the ability to synthesize a substance or substances present in whole serum, but not present in the serum fractions 1B, 2A and 3B.

In terms of population genetics, a single mutant which requires more serum components than its parent, may well have a shorter generation time than its parent as it has less energy requiring synthetic activity to perform. Other things being equal, it would in fact have to have a shorter generation time if this mutant were to outgrow its parent. Comparison of data on generation times of Giardia in M5 medium during the 6 month and 3 month periods supports this hypothesis. The average generation time in medium containing whole serum for 24 separate experiments during the 6 month period was 16.9 $\pm$ 1.1 hour while the average generation time for 10 experiments in the 3 month period was 13.8 $\pm$ 1.0 hour. A standard "t" test (119), performed on these 2 average generation times, showed that they were significantly different at the 0.05 level of probability. Comparison by "t" test of the 16.9 $\pm$ 1.1 hour value to the 18.1 $\pm$ 1.6 hour generation time, measured in 8 experiments

at the beginning of this thesis project, showed these 2 values to be not significantly different at the 0.05 probability level. So, the only observed statistically significant change in generation time occurred during the same 3 month period in which Giardia was losing its ability to grow in medium containing 1B+2A or 1B+2A + 3B.

In a review, Honigberg (120) noted that trypanosomes of the evansi subgroup occur in one morphological form (the same form as that of brucei trypanosomes in mammals), and have no cyclic development in insect intermediates. He suggests that the change observed in the brucei trypanosomes during animal passage (118) may reflect the natural evolution of brucei to evansi trypanosomes. Parasite evolution in general has often been described as the adaptation of an originally free-living form to a dependence on its host (121, 122). One form of this evolution is the loss of synthetic capabilities by the organism as continued existence in the host environment provides those necessary materials that the parasite once had to synthesize.

It seems likely that the observed change in Giardia growth requirements demonstrates a piece of "laboratory evolution" in which the organism, maintained in vitro on a richer medium than occurs in vivo, simultaneously lost some of its synthetic ability and in the process gained a competitive growth advantage over the parent strain.

SUMMARY AND CONCLUSIONS

The nature of the contribution of human serum to the growth of *Giardia* from the rabbit was studied. Two serum fractions could successfully replace serum in support of parasite growth. One of these fractions was of relatively low molecular weight, the other of higher molecular weight.

The low molecular weight fraction was replaceable with a commercial preparation of 4x crystallized human albumin. The higher molecular weight fraction was compared by several analytical methods with those serum fractions not capable of supporting the protozoan's growth. Significantly, the higher molecular weight fraction was rich in IgA, IgG and haptoglobin while the others were not. Subsequent experiments with heat treated and abnormal sera suggested that the significant component of the higher molecular weight fraction was IgA.

A statistically valid method has been developed of determining numbers of *Giardia* trophozoites in culture tubes. The method permitted the study of many more variables than would otherwise have been possible.

The number of generations per culture and the generation time of *Giardia* in medium containing whole serum were measured; these values compared favorably to values reported in the literature on other *Giardia* culture systems.

Towards the end of this work, *Giardia* trophozoites, serially maintained on serum-containing medium in stock culture were observed over a period of months to undergo a decreased ability to grow in medium containing the low and high molecular weight serum fractions, and

simultaneously to develop a shorter generation time in complete medium. This suggested that a mutant had occurred in stock culture and had overgrown the parent population of trophozoites.

REFERENCES

1. Meyer, E. A. & Pope, B.L. Culture *in vitro* of *Giardia* trophozoites from the rabbit and chinchilla. Nature, 1965. 207, 1417-1418
2. Brandborg, L. L., Tankersley, C.B., Gottlieb, S., Barancik, M. & Sartor, V.E. Histological demonstration of mucosal invasion by *Giardia lamblia* in man. Gastroenterology, 1967. 52, 143-150
3. Morecki, R. & Parker, J.G. Ultrastructural studies of the human *Giardia lamblia* and subjacent jejunal mucosa in a subject with steatorrhea. Gastroenterology, 1967. 52, 151-164
4. Barbieri, D., DeBrito, T., Hoshino, S., Nascimento F[a], O. B., Martins Campos, J.V., Quarentei, G. & Marcondes, E. Giardiasis in childhood. Arch. Dis. Child., 1970. 45, 466-472
5. Faust, E. C. & Russell, P.F. Craig and Faust's Clinical Parasitology. Philadelphia: Lea and Febiger, 1964. (page 93)
6. Belding, D. L. Textbook of Parasitology (Third edition). New York: Appleton-Century-Crofts, 1965. (pages 125-126)
7. Cantor, D., Biempica, L., Toccalino, H. & O'Donnel, J.C. Small intestine studies in Giardiasis. Am. J. Gastroenterol., 1967. 47, 134-141
8. Alp, M. H. & Hislop, I. G. The effect of *G. lamblia* infestation of the gastro-intestinal tract. Australian Annals of Medicine, 1969. 18, 223-237
9. Karapetyan, A. E. A method of cultivation of *Giardia duodenalis*. Tsitologiia, 1960. 2, 379-384

10. Karapetyan, A. E. In vitro cultivation of Giardia duodenalis. J. Parasitol., 1962. 48, 337-340

11. Meyer, E. A. Isolation and axenic cultivation of Giardia trophozoites from the rabbit, chinchilla and cat. Exp. Parasitol., 1970. 27, 179-183

12. Fell, H. B. & Mellanby, E. Metaplasia produced in cultures of chick ectoderm by high vitamin A. J. Physiol. (London), 1953. 119, 470-488

13. Raisz, L. G. Effect of calcium on parathyroids in organ culture. Fed. Proc., 1962. 21, 207 (Abstract)

14. Khouw, B. T. & McCurdy, H. G. Jr. Nutritional studies of a flagellated protozoan Hexamita inflata from the Canadian oyster Crassotrea virginica. Can. J. Microbiol., 1968. 14, 817-821

15. Schultze, H. E. & Heremans, J. F. Molecular biology of human proteins. I. Nature and metabolism of extracellular proteins. Amsterdam: Elsevier, 1966.(pages 773-809)

16. Ito, T. & Moore, G. E. The growth-stimulating activity of peptides on human hemopoietic cell culture. Exp. Cell Res., 1969. 56, 10-14

17. Higgins, M. L., Tillman, M. C., Rupp, J. P. & Leach, F. R. The effect of polyamines on cell culture cells. J. Cell Physiol., 1969. 74, 149-154

18. Temin, H. M. Studies on carcinogenesis by avian sarcoma virus. J. Cell Physiol., 1967. 69, 377-384.

19. Rajkovic', A. D. Beef serum as a supplement to media for the cultivation of the Noguchi strain of Treponema pallidum. Z. Med. Mikrobiol. Immunol., 1966. 152, 100-111

20. Pollack, M. R. The growth of H. pertussis on media without blood. Br. J. Exp. Pathol., 1947. 28, 295-307

21. Johnson, R. C. & Wilson, J. B. Nutrition of Leptospira pomona. J. Bacteriol., 1960. 80, 406-411

22. Taylor, A. E. R. & Baker, J. R. The cultivation of parasites in vitro. England: Blackwell, 1968

23. Sinha, D. P. & Hopkins, C. A. In vitro cultivation of the tapeworm H. nana from larva to adult. Nature, 1967. 215,1275-1276

24. Levine, H. S. & Silverman, P. H. Cultivation of Ascaris suum larvae in supplemented and unsupplemented chemically defined media. J. Parasitol., 1969. 55, 17-21

25. Diamond, L. S. Techniques of axenic cultivation of Entameba histolytica Schaudinn, 1903 and E. histolytica-like amebae. J. Parasitol., 1968. 54, 1047-1056.

26. Diamond, L. S. The establishment of various trichomonads of animals and man in axenic cultures. J. Parasitol., 1957. 43, 488-490.

27. Glaser, R. W. & Coria, N. A. The partial purification of Balantidium coli from swine. J. Parasitol., 1935. 21, 190-193

28. Jainchill, J. L. & Todaro, G. J. Stimulation of cell growth in vitro by serum with and without growth factor. Exp. Cell Res., 1970. 59, 137-146

29. Frank, W. & Zabel, S. Über die Bedeutung von Kälberserum für Kulturen embryonaler Rattenzellen. Exp. Cell Res., 1970. 59, 186-192

30. Dulbecco, R. Topoinhibition and serum requirement of transformed and untransformed cells. Nature, 1970. 227, 802-806

31. Clarke, G. D., Stoker, M. G. P., Ludlow, A. & Thornton, M. Requirement of serum for DNA synthesis in BHK-21 cells: effects of density, suspension and virus transformation. Nature, 1970. 227, 798-801

32. Vogt, A., Mishell, R. J. & Dutton, R. W. Stimulation of DNA synthesis in cultures of mouse spleen cell suspensions by bovine transferrin. Exp. Cell Res., 1969. 54, 195-200

33. Sefton, B. M. & Rubin, H. Release from density dependent growth inhibition by proteolytic enzymes. Nature, 1970. 227, 843-845

34. Cooperband, S. R., Bondevik, H., Schmid, K. & Mannick, J. A. Transformation of human lymphocytes: inhibition by homologous globulin. Science, 1968. 159, 1243

35. Agrell, I. P. S. & Karlson, B. W. The action of phytohemagglutinin upon mitotic inhibitors in the nutrient medium for free-living amebae. Exp. Cell Res., 1967. 48, 634-640

36. Gyévai, A. & Fazekas, I. Changes in the histochemical behavior of cultured cells due to differences in the serum component of the medium. Acta Histochem., 1969. 33, 188-190

37. Smull, C. E. & Ludwig, E. H. Delay of poliovirus plaque formation with use of fresh bovine serum in growth medium of host cells. Appl. Microbiol., 1969. 17, 449-453

38. Bailey, J. M. Lipid metabolism in cultured cells. VI. Lipid biosynthesis in serum and synthetic growth media. Biochim. Biophys. Acta, 1966. 125, 226-236

39. Burns, C. H. & Rothblat, G. H. Cholesterol excretion by T. C. cells: effect of serum lipids. Biochim. Biophys. Acta, 1969. 176, 616-625

40. Adamson, L. F. & Anast, C. S. Amino acid, potassium and sulfate transport and incorporation by embryonic chick cartilage: the mechanism of the stimulating effects of serum. Biochim. Biophys. Acta, 1966. 121, 10-20

41. Hijmans, J. C. & McCarty, K. S. Effect of hydrocortisone on chick duodenum cultured in chemically defined medium. Proc. Soc. Exp. Biol. Med., 1969. 131, 1407-1412

42. Fisher, M. W., Puck, T. T. & Sato, G. Molecular growth requirements of single mammalian cells: the action of fetuin in promoting cell attachment to glass. Proc. Natl. Acad. Sci. USA, 1958. 44, 4-10

43. Lieberman, I. & Ove, P. Growth factors for mammalian cells in culture. J. Biol. Chem., 1959. 234, 2754-2758

44. Holmes, R. Preparation from human serum of an alpha-one protein which induces the immediate growth of unadapted cells in vitro. J. Cell Biol., 1967. 32, 297-308

45. Stalheim, O. H. V. & Wilson, J. B. Antigenicity and immunogenicity of leptospires grown in chemically characterized medium. Am. J. Vet. Res., 1964. 25, 1277-1280

46. Mickelson, M. N. & Slade, H. D. Absence of type specific M antigen from group A streptococci grown in a chemically defined medium. J. Bacteriol., 1964. 87, 1251

47. Sollod, A. E. & Soulsby, E. J. L. Cultivation of Trypanosoma theilerei at 37 C in partially defined media. J. Protozool., 1968. 15, 463-470

48. Oelerich, S. & Mannweiler, E. Über den Einflusz des Nährmediums auf die antigenen Eigenschaften von Trichomonas vaginalis. II. Mitteilung Serumfreies medium. Zentralbl. Bakteriol. (Orig.), 1968. 207, 552-559

49. Birch, J. R. & Pirt, S. J. The choline and serum protein requirements of mouse fibroblast cells (strain LS) in culture. J. Cell Sci., 1969. 5, 135-142

50. Michl, J. Metabolism of cells in tissue culture in vitro. I. The influence of serum protein fractions on the growth of normal and neoplastic cells. Exp. Cell Res., 1961. 23, 324-334

51. Jenkin, H. M. & Anderson, L. E. The effect of oleic acid on the growth of monkey kidney cells (LLC-MK2). Exp. Cell Res., 1970. 59, 6-10

52. Myers, W. F., Cutler, L. D. & Wisseman, C. L. Jr. Role of erythrocytes and serum in the nutrition of Rickettsia quintana. J. Bacteriol., 1969. 97, 663-666

53. Pollack, M. R. The growth of H. pertussis on media without blood. Br. J. Exp. Pathol., 1947. 28, 295-307

54. Shorb, M. S. & Lund, P. G. Requirement of trichomonads for unidentified growth factors, saturated and unsaturated fatty acids. J. Protozool., 1962. 6, 122-130

55. Fisher, H. W., Puck, T. T. & Sato, G. Molecular growth requirements of single mammalian cells. III. Quantitative colonial growth of single S3 cells in a medium containing synthetic small molecular constituents and two purified protein fractions. J. Exp. Med., 1959. 109, 649-659

56. Socransky, S. S. & Hubersak, C. Replacement of ascitic fluid or rabbit serum requirement of Treponema dentium by α-globulin. J. Bacteriol., 1967. 94, 1795-1796

57. Malagón, F. & Venkatesan, G. & S. Uptake of serum γ globulin in culture by trophozoites of Entameba histolytica. Trans. R. Soc. Trop. Med. Hyg., 1970. 64, 18 (Abstract)

58. Holley, R. W. & Kiernan, J. A. "Contact inhibition" of cell division in 3T3 cells. Proc. Natl. Acad. Sci. USA, 1968. 60, 300-304

59. Burger, M. M. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. Nature, 1970. 227, 170-171

60. Sato, G., Fisher, H. W. & Puck, T. T. Molecular growth requirements of single mammalian cells. Science, 1957. 126, 961-964

61. Latour, N. G., Reeves, R. E. & Guidry, M. A. Steroid requirement of E. histolytica. Exp. Parasitol., 1965. 16, 18-22

62. Rajkovic', A. D. Cultivation of the Noguchi strain of Treponema pallidum in a lipid medium in the absence of serum. Z. Med. Mikrobiol., Immunol., 1967. 153, 297-312

63. Rodwell, A. W. A defined medium for Mycoplasma strain Y. J. Gen. Microbiol., 1969. 58, 39-47

64. Lesser, E. Replacement of serum in the cultivation of H. meleagridis. J. Parasitol., 1960. 46, 271

65. Ellinghausen, H. C. & McCullough, W. G. Nutrition of Leptospira pomona and 13 other serotypes: Fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Am. J. Vet. Res., 1965. 26, 45-51

66. Ham, R. G. Albumin replacement by fatty acids in clonal growth of mammalian cells. Science, 1963. 140, 802-803

67. Siddiqui, W. A., Schnell, J. V. & Geiman, Q. M. Stearic acid as plasma replacement for intracellular in vitro culture of Plasmodium knowlesi. Science, 1967. 156, 1623-1625

68. Bone', G. J. & Parent, G. Stearic acid, an essential growth factor for Trypanosoma cruzi. J. Gen. Microbiol., 1963. 31, 261-266

69. Ham, R. G. An improved nutrient solution for diploid Chinese hamster and human cell lines. Exp. Cell Res., 1963. 29, 515-526

70. Ham, R. G. Putrescine and related amines as growth factors for a mammalian cell line. Biochem. Biophys. Res. Commun., 1964. 14, 34-38

71. Ham, R. G. Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proc. Natl. Acad. Sci. USA, 1965. 53, 288-293

72. Johnson, R. C. & Gary, N. D. Nutrition of L. pomona. I. A chemically defined substitute for rabbit serum ultrafiltrate. J. Bacteriol., 83, 668-672

73. Shenberg, E. Growth of pathogenic Leptospira in chemically defined media. J. Bacteriol., 1967. 93, 1598-1606

74. Lincicome, D. R. Growth factor in normal rat serum for T. lewisi. J. Parasitol., 1955. 41, 15

75. Lincicome, D. R. Growth of T. lewisi in the heterologous mouse host. Exp. Parasitol., 1958. 7, 1-13

76. Lincicome, D. R. & Francis, E. H. Quantitative studies on heterologous sera inducing development of Trypanosoma lewisi in mice. Exp. Parasitol., 1961. 11, 68-76

77. Greenblatt, C. L., Jori, L. A. & Cahnmann, H. J. Chromatographic separation of a rat serum growth factor required by T. lewisi. Exp. Parasitol., 1969. 24, 228-242

78. Neuman, R. E. & Tytell, A. A. Development of serumless medium. Proc. Soc. Exp. Biol. & Med., 1960. 104, 252-256

79. Rigas, D. A. An automatic pressure-vacuum ultrafiltration apparatus. In preparation.

80. Fahey, J. L. & McLaughlin, C. Preparation of antisera specific for 6.6S γ-globulins, β-2A globulins, γ_1-macroglobulins and for type I and II common γ-globulin determinants. J. Immunol., 1963. 91, 484-497

81. Campbell, D. H., Garvey, J. S., Cremer, N. E. & Sussdorf, D. H. Methods in Immunology. New York: W. A. Benjamin, 1964. (pages 149-155)

82. Campbell, D. H., Garvey, J. S., Cremer, N. E. & Sussdorf, D. H. ibid. (pages 143-149)

83. E-C Apparatus Corporation Bulletin, Philadelphia. Electrophoresis and Countercurrent, 1966.

84. Davis, B. J. Disc-electrophoresis - II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci., 1964. 121, 404-427

85. Waddell, W. J. A simple ultraviolet spectrophotometric method for the determination of protein. J. Lab. Clin. Med., 1956. 48, 311-314

86. Murphy, J. B. & Kies, M. W. Note on spectrophotometric determination of proteins in dilute solutions. Biochim. Biophys. Acta, 1960. 45, 382-384

87. Mancini, G., Carbonara, A. O. & Heremans, J. F. Immunochemical quantitation of antigens by single radial diffusion. Immunochem., 1965. 2, 235-254

88. Hanks, J. H. & Wallace, R. E. Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exp. Biol. Med., 1949. 71, 196-200

89. Evans, V. J., Bryant, J. C., Kerr, H. A. & Schilling, E. L. Chemically defined media for cultivation of long-term cell strains from four mammalian species. Exp. Cell Res., 1964. 36, 439-474

90. Brandtzaeg, P., Fjellanger, I. & Gjeruldsen, S. T. Human secretory immunoglobulins. I. Salivary secretions from individuals with normal or low levels of serum immunoglobulins. Scand. J. Haematol., 1970. Supplementum 12, 34

91. Schultze, H. E. & Heremans, J. F. Molecular biology of human proteins. I. Nature and metabolism of extracellular proteins. Amsterdam: Elsevier, 1966. (pages 180-181)

92. Meyer, E. A. & Chadd, J. A. Preservation of Giardia trophozoites by freezing. J. Parasitol., 1967. 53, 1108-1109

93. Karapetyan, A. E. Determination of the growth rate of Lamblia intestinalis cultivated in vitro. Acta Protozool. (Warsaw), 1963. 1, 321-326

94. Roux, G. & Ecalle, R. Influence du suc pancreatique sur la proliferation in vitro de Giardia duodenalis. C. R. Acad. Sci. [D] (Paris), 1968. 266, 2434-2436

95. Ecalle, R. Influence de quelques enzymes de suc pancretique sur la culture in vitro de Giardia duodenalis. C. R. Soc. Biol. (Paris) 1968. 162, 2321-2323

96. Schultze, H. E. & Heremans, J. F. Molecular biology of human proteins. I. Nature and metabolism of extracellular proteins. Amsterdam: Elsevier, 1966. (page 3)

97. Schultze, H. E. & Heremans, J. F. ibid. (page 297)

98. Schultze, H. E. & Heremans, J. F. ibid. (pages 246-247)

99. Dixon, F. J. & Maurer, P. H. Immunologic unresponsiveness induced by protein antigens. J. Exp. Med., 1955. 101, 245-257

100. Linscott, W. D. Contamination of commercial rabbit albumin preparation by bovine albumin. Science, 1963. 142, 1170-1172

101. Schaer, J. C. & Schindler, R. The requirement of mammalian cell culture for serum proteins. Growth-promoting activity of pepsin-digested serum albumin in different media. Biochim. Biophys. Acta, 1967. 147, 154-161

102. Tritsch, G. L., Floss, D. R. & Moore, G. E. Protein utilization by cultured mammalian cells: the fate of guanidinated serum albumin. Exp. Cell Res., 1966. 42, 523-536

103. Ryser, H. J-P. The measurement of I^{131} serum albumin uptake by tumor cells in tissue culture. Lab. Invest., 1963. 12, 1009-1017

104. Chen, R. F. Removal of fatty acids from serum albumin by charcoal treatment. J. Biol. Chem., 1967. 242, 173-181

105. Goodman, D. S. Preparation of human serum albumin free of long-chain fatty acids., Science, 1957. 125, 1296-1297

106. Tomasi, T. B., Tan, E. M., Solomon, A. & Prendergast, R. A. Characteristics of an immune system common to certain external secretions. J. Exp. Med., 1965. 121, 101-124

107. Plaut, A. G. & Keonil, D. Immunoglobulins in human small intestinal fluid. Gastroenterology, 1969. 56, 522-530

108. Crabbe', P. A. & Heremans, J. F. The distribution of immunoglobulin-containing cells along the human gastrointestinal tract. Gastro-enterology, 1966. 51, 305-316

109. Tomasi, T. B. Human immunoglobulin A. New England J. Med., 1968. 279, 1327-1330

110. Collins-Williams, C., Lamenza, C. & Nizami, R. Immunoglobulin IgA: A review of the literature. Ann. Allergy, 1969. 27, 225-231

111. Wales, E. E. Jr., Englert, E. Jr., Winward, R. T., Maxwell, J. G. & Stevens, L. E. Disc electrophoresis-immunodiffusion of serum proteins in normal human gallbladder bile. Proc. Soc. Exp. Biol. Med., 1969. 132, 146-149

112. Honigberg, B. M. Comparative pathogenicity of Trichomonas vaginalis and Trichomonas gallinae to mice. I. Gross pathology quantitative evaluation of virulence, and some factors affecting pathogenicity. J. Parasitol., 1961. 47, 545-571

113. Lindgren, R. D. & Ivey, M. H. The effect of cultivation and freezing on the virulence of Trichomonas vaginalis for mice. J. Parasitol., 1964. 50, 226-229

114. Stabler, R. M., Honigberg, B. M. & King, V. M. Effect of certain laboratory procedures on virulence of the Jones' Barn strain of Trichomonas gallinae for pigeons. J. Parasitol., 1964. 50, 36-41

115. Honigberg, B. M., Stabler, R. M., Livingston, M. C. & Kulda, J. Further observations on the effects of various laboratory procedures on the virulence of Trichomonas gallinae for pigeons. J. Parasitol., 1970. 56, 701-708

116. Dwyer, D. M. & Honigberg, B. M. Effect of certain laboratory procedures on the virulence of Histomonas meleagridis for turkeys and chickens. J. Parasitol., 1970. 56, 694-700

117. Vincent, P. & Neal, R. A. Duration of invasiveness of Entameba histolytica maintained in vitro. Parasitol. (London), 1960. 50, 449-452

118. Trager, W. Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. Nature, 1965. 208, 762-766

119. Goldstein, A. Biostatistics. An introductory text. New York: Macmillan, 1964. (pages 51-55)

120. Honigberg, B. M. Some recent advances in our understanding of the parasitic protozoa. J. Protozool., 1968. 15, 223-230

121. Belding, D. L. Textbook of Parasitology (third edition). New York: Appleton-Century-Crofts, 1965. (pages 8-10)

122. Markell, E. K. & Voge, M. Medical Parasitology. Philadelphia: W. B. Saunders, 1965. (pages 9-10)

APPENDIX

A. Preparation of M5 Medium

Component A

1. 65 ml Hanks' balanced salt solution. This was prepared according to the procedure of Hanks and Wallace (88) and kept at 5 C until use.
2. 1 gm yeast extract (Difco, Detroit, Mich.).
3. 0.1 gm L (+) cysteine-HCl (Sigma, St. Louis, Mo.).

The yeast extract and cysteine-HCl were mixed into the Hanks' solution and the mixture autoclaved at 121 C for 15 min.

Component B

1. 5 ml sterile medium NCTC-135 (Gibco, Grand Island, N.Y.).
2. 25 ml Seitz-filtered, complement inactivated (56 C for 30 min) human serum. The sterile inactivated serum was stored frozen at -20 C and thawed in a 37 C water bath just before use.
3. 0.5 ml potassium penicillin G (100,000 units/ml).
4. 0.25 ml streptomycin sulfate (0.34 gm/ml).

Reducing Solution

1. 0.05 gm reduced glutathione (Calbiochem, L. A. Calif.).
2. 0.05 gm cysteine-HCl.
3. 5 ml Hanks' balanced salt solution.
4. 0.75 ml N NaOH.

This solution was Millipore-filtered.

Components A and B and reducing solution were prepared and mixed together just before use, and the pH adjusted to 6.7 $\pm$ 0.05 with 7 1/2% $NaHCO_3$ and/or 0.1N HCl.

B. Formulas for % Activity and Specific Activity

G_T Number of generations occurring in a particular experiment in test medium.

G_{WS} Number of generations in whole serum control medium.

G_0 Number of generations in medium with no serum and no serum substitute.

C Concentration in gm/ml of serum protein or serum fraction protein in medium.

$$\% \text{ Activity} = \frac{G_T - G_0^*}{G_{WS} - G_0^*} \times 100$$

$$\text{Specific Activity} = \frac{G}{C}$$

* The G_0 term was omitted as we have never seen Giardia grow in serumless medium.