STUDIES OF THE *IN VITRO* GROWTH REQUIREMENTS OF *GIARDIA*

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INTRODUCTION

A. HISTORICAL ADVANCES IN THE ROLE OF *GIARDIA* IN DISEASE

Giardia in Man

Giardia was discovered and described in human stools by Leeuwenhoek in 1681 and later was named after Lambl, who rediscovered it in 1859 (16). *Giardia* occur in trophozoite and cyst forms. Trophozoites have four pairs of flagella and a sucking disk, with which they attach to the mucosa of the duodenum and upper jejunum. Multiplication of trophozoites is by binary fission; encystment occurs as they are swept down the gastrointestinal tract. The cyst form is responsible for transmission from host to host, and if maintained in a moist environment, will survive for prolonged periods.

The incidence of *Giardia* infection in this country varies from 1.6 to 20 % (2, 15, 27, 29, 34). The average world incidence has been estimated at approximately 10 % (8).

Since the discovery of quinacrine as an effective therapeutic agent for giardiasis in 1937, numerous clinical reports in the literature consider certain symptoms to result from the presence in the small intestine of large numbers of these parasites. It is convenient to think of *Giardia* as the etiologic agent for a spectrum of illnesses rather than a specific disease. At one extreme is the asymptomatic carrier, while the full-blown malabsorption syndrome of children represents the other. In between fall the pleomorphic and commonly observed group with symptoms of epigastric

abdominal pain, diarrhea, anorexia, nausea and vomiting, flatulence, malaise, melena, gall bladder colic, jaundice, malnutrition and constipation (4, 6, 7, 18, 22, 27, 28, 31, 32, 34, 36, 47, 48, 49, 50, 51). These symptoms were eradicated by treatment of the giardiasis with quinacrine in 95 % of the cases.

Miller (31) in 1926 reported the first case of celiac-like disease due to giardiasis. Katsampes et al. (22), Veghelyi (49, 51), Court and Anderson (7) and Cortner (6) have reported giardiasis in children producing a celiac syndrome with clinically manifest steatorrhea and malabsorption. Thorough investigation of these cases revealed no pathogens other than Giardia. Veghelyi (50) postulates that Giardia causes malabsorption by mechanical obstruction of the mucosal surfaces of the duodenum and jejunum.

Contradictory evidence is presented by Palumbo et al. (37) who reviewed 69 cases of giardiasis having laboratory data relating to intestinal absorptive function. Evaluation of these patients using radioactive cobalt-labeled vitamin B_{12} absorption tests and response to quinacrine chemotherapy revealed no conclusive evidence that Giardia caused a malabsorption syndrome; however, it could not be excluded as an etiologic agent in 2 cases. The authors concluded that their data indicated finding Giardia in the gastrointestinal tract was concidental. This conclusion is also in agreement with that of Monat and McKinney (32) who, since 1946 have regarded Giardia as a non-pathogen often found in disease due to other causes.

Lyon and Swalm (27) reported that jaundice and gall bladder colic are caused by Giardia. They postulate obstruction of bile ducts by massive numbers of trophozoites or irritation and edema at the ampulla of Vater resulting from attachment of trophozoites to the adjacent mucosa as the mechanism of pathogenesis. McGowan (34) lists 8 cases of cholecystitis presumably due to Giardia in which cysts and or trophozoites were found in the gall bladder or bile.

Da Silva (9) in a recent biopsy study of the duodenum and jejunum of patients with giardiasis has shown that trophozoites are found in mucous strands adherent to the mucosal surfaces. No specific tissue pathology was reported. There was no evidence that trophozoites invade the mucosa.

Rendtorff (42) in an attempt to satisfy Koch's postulates, was able to infect human volunteers with Giardia by feeding them capsules containing cysts. All uninfected volunteers receiving 10,000 or more cysts were infected and began excreting cysts in their stools. No specific symptoms could be attributed to Giardia, and 86 % of patients infected showed a spontaneous disappearance of Giardia in 5 to 41 days.

Most of the 10 % of the world population, then, are probably parasitized by numbers of Giardia not large enough to produce disease. It is when the organisms are able to multiply in massive numbers that disease results. Failure of preliminary experiments to satisfy Koch's postulates need not be considered conclusive evidence that Giardia does not cause disease. A knowledge of the *in vitro* requirements of Giardia may well

provide insight into the conditions under which these parasites are able to cause disease.

Giardia in Animals

Hartman and Kyser (18), and Hegner (19, 20, 21) state that Giardia species are found in mammals, reptiles, amphibians, birds, fish, and in the intestine of nematodes of South America. Nomenclature and classification of Giardia species from the many hosts has been fraught with confusion. The work of Hegner (20), Simon (45, 46), Filice (14) and Kofoid and Christiansen (26) on the classification of Giardia according to external dimensions and internal structure of trophozoites has clarified much of the confusion. Filice (14) recognizes three species of Giardia each of which contain several races. Giardia agilis, commonly found in tadpoles and frogs, Giardia muris, found in certain species of rats and mice, and Giardia duodenalis, which consists of some 10 races including Giardia from rabbits, man, chinchilla and several species of rats and mice. Each race has been given a name and Giardia are assigned a race on the basis of average dimensions.

Several workers (42, 43, 44, 45) have collected evidence to suggest that Giardia species are host specific.

Shelton (44) was able to produce intestinal disturbances and abnormal fecal eliminations in the chinchilla by infecting parasite free animals with Giardia cysts. Death did occur in his infected animals, but complicating bacterial or intercurrent parasitic infections were found to be predisposing

factors. He makes reference to several reports of Giardia as the cause of gastrointestinal disturbances in the chinchilla.

The incidence of Giardia in animals increases with poor sanitary conditions among other factors. However, even in the face of dirty housing and improper care there is little to suggest Giardia as the cause of a specific disease in animals other than the chinchilla.

Hindrance to Giardia Research

For over one hundred years the lack of an adequate technique for in vitro cultivation of Giardia loomed as the obstacle to further study of this parasite. Reports by Chatterjee (5) in 1927, Penso (40) in 1929 and Poindexter (41) in 1935 of the in vitro growth of trophozoites in various bacteriological media were encouraging, but could not be duplicated by other workers. Trophozoites reportedly survived from 11 to 35 days after inoculation, and were passed through several generations before disappearing from the culture tubes.

Interest in Giardia in this area was stimulated by an outbreak of gastroenteritis in 1954 and 1955. The only common denominator in several thousand cases was the presence of Giardia cysts and or trophozoites in stool specimens.

In 1960, Karapetyan (24) reported a technique for the in vitro cultivation of Giardia trophozoites obtained from the duodenum of a patient. This

technique utilized a complex medium, chicken fibroblast cultures, and Candida guilliermondi. The Giardia attached themselves to the fibroblasts where they multiplied rapidly. The tissue cultures were gradually destroyed, while the trophozoites and fungi continued to grow. The trophozoites failed to grow in the absence of the Candida. A modification of this technique for the in vitro cultivation of trophozoites was reported by Karapetyan (25) in 1962. Trophozoites from rabbits were grown in a similar complex medium to which Saccharomyces cerevisiae had been added at the time of inoculation. No fibroblast cultures were used in this technique; the trophozoites, reportedly, grew rapidly and attached themselves to the inner surface of glass bottles. It is these studies which will be confirmed and extended, thereby marking the beginning of scientific investigation of Giardia species several hundred years after their original discovery.

B. OBJECTIVES OF THIS STUDY

Initial Objectives

Prior to the work of Karapetyan, the objectives of this study were as follows:

1. To devise a culture medium for the in vitro cultivation of Giardia trophozoites from various animal species.
2. To cultivate in vitro, trophozoites obtained from man.

3. To study the *in vitro* growth characteristics of various species of *Giardia* maintained in culture.

Final Objectives

After the report by Karapetyan, the objectives were revised and were as follows:

1. To establish and maintain *in vitro*, an axenic culture of *Giardia* trophozoites obtained from the rabbit and chinchilla.
2. To cultivate *Giardia* trophozoites from the duodenum of man *in vitro* using the technique of Karapetyan.
3. To observe the *in vitro* growth characteristics of *Giardia* trophozoites maintained in culture.

MATERIALS AND METHODS

A. MEDIA

Karapetyan's Medium

The basal liquid medium employed for isolation and maintenance of cultures was that initially advocated by Karapetyan (24) in 1960, which consists of 60% Hanks' balanced salt solution, 25% inactivated filtered human serum, 10% Hottinger's digest (a tryptic meat digest), and 5% chick embryo extract. The medium was adjusted to pH 7.2 to 7.4 by passing CO_2 through the fluid, penicillin (500 units per ml) and streptomycin (250 units per ml) were then added. Sterile medium was stored at 5 C in bottles with serum-type stoppers in 200 ml aliquots. Strict aseptic technique was observed when withdrawing medium from bottles.

Hanks' balanced salt solution was prepared in 20 X concentration using the method described by Paul (38), as described in TABLE I.

Pooled human serum was obtained from the Serology Division of the Clinical Pathology Laboratory of the University of Oregon Medical School. After inactivation in a 56 C water bath for 30 min the serum was sterilized by passage through a washed Seitz filter and stored in 50 ml aliquots at minus 15 C until used.

Chick embryo extract was prepared using a modification of the method described by Paul (39), as described in detail in TABLE II. Extract, stored

in 10 ml aliquots at minus 15 C, was slowly thawed and centrifuged at 2500 rpm for 15 min prior to use.

The method of preparing Hottinger's digest is described by Karapetyan (25), details are presented in TABLE III.

Tissue Culture Media

In an effort to substitute a chemically defined medium for Karapetyan's other standard tissue culture media were utilized as substitutes or in combination with components of Karapetyan's medium as follows:

Eagle's Medium

Medium 199

Medium NCTC 107

Medium NCTC 109

These media were obtained commercially (Colorado Serum Co.), the ingredients are given in TABLES IV, V, VI AND VII.

Substituted Components in Media

Horse and rabbit serum (Microbiological Associates) were substituted in equal concentrations for human serum in Karapetyan's medium. Serum fractions such as crystalline bovine serum albumin, fraction V human serum albumin (Nutritional Biochemicals Corp.), and human serum albumin (Red Cross Blood Bank) were substituted in concentrations equal to that found in whole human serum.

Bacto-Peptone, Bacto-Tryptone (Difco), and Trypticase (Baltimore Biological Laboratories) were substituted for Hottinger's digest in Karapetyan's medium. These preparations were used in concentrations of 10 to 50 mg per ml final concentration. Sterilization was accomplished by autoclaving in 10% solutions at 121 C and 15 psi for 10 min.

Sodium acetate (Baker Chemicals), sodium pyruvate, succinic acid and alpha ketoglutaric acid (Nutritional Biochemicals Corp.) were substituted for glucose in equal concentrations (20 gm per liter) in Hanks' solution, and subsequently in Karapetyan's medium. The method as described was used to prepare solutions, and pH was adjusted to 7.0 with 0.1 N sodium hydroxide prior to using in media.

B. CULTURE APPARATUS

Tubes, Stoppers and Racks

Leighton type 16 X 125 mm tubes with silicone rubber stoppers (Bellco Glass, Inc.) and disposable constricted type 16 X 125 mm tubes with latex rubber stoppers (Demuth Glass Co.) were used. Tubes were stored and incubated in stainless steel tube racks (A. H. Thomas Co.) slanted at a 5 to 7 degree angle. Tubes and racks are illustrated in Figure 1.

Incubator

Cultures were incubated in a standard air jacket incubator (National Appliance Co.) maintained at 37 C.

C. YEAST AND PRODUCTS SUBSTITUTED FOR YEAST

Yeast Cultures

Cultures of *Saccharomyces cerevisiae* were obtained from the stock culture collection of the Department of Bacteriology of the University of Oregon Medical School. Cultures were inoculated on Sabouraud's dextrose agar, and were incubated at 23C. Daily transfer of cultures provided a continuous source of fresh viable yeast. Once monthly new cultures were inoculated from stock yeast cultures and all old cultures were discarded. To inoculate *Giardia* cultures with fresh yeast, one loopful of *S. cerevisiae* was suspended in 1 ml Karapetyan's medium, and 1 or 2 drops of suspension were added to the cultures.

Bacterial Cultures

Cultures of *Escherchia coli* and *Streptococcus faecalis* were obtained from the stock culture collection of the Department of Bacteriology of the University of Oregon Medical School. *E. coli* cultures were inoculated on extract agar and incubated at 37C for 24 hr. 1 drop of a suspension consisting of a small loopful of *E. coli* per ml of antibiotic-free Karapetyan's medium was added to *Giardia* cultures. *S. faecalis* cultures were inoculated in nutrient broth medium and incubated at 37C for 48 hr. 1 drop of a suspension consisting of a small loopful of *S. faecalis* per ml of antibiotic-free Karapetyan's medium was added to *Giardia* cultures.

Yeast Products

Heat Killed Yeast

Heat killed yeast was prepared by suspending 1 loopful of S. cerevisiae per ml of Hanks' solution and autoclaving at 121 C and 15 psi for 10 min. Cells failed to grow on Sabouraud's agar after 24 hr incubation at 23 C.

Yeast Autolysate

Yeast Autolysate (Albimi Laboratories Inc.) was prepared in a concentration of 10 gm per 100 ml distilled water. This solution was autoclaved at 121 C and 15 psi for 10 min. Sterilization was assumed if no bacterial growth was obtained in either extract agar or nutrient broth medium after 24 hr incubation at 37 C.

Yeast Extract

Yeast Extract (Difco) was prepared in a concentration of 10 gm per 100 ml distilled water. Sterilization by autoclaving at 121 C and 15 psi for 10 min was assured using the media for bacterial growth as above.

Yeast Homogenate

Yeast homogenate was prepared from a suspension of S. cerevisiae in Hanks' solution using either a high speed homogenizer (Sorvall Omni-Mixer, I. Sorvall Corp.) and glass beads (Superbrite, Type 113, Minnesota Mining and Manufacturing Co.) or a hydraulic pressure chamber (Dr. H. Taylor, Department of Bacteriology, Portland State College). Large numbers

of S. cerevisiae were obtained by inoculating Sabouraud's liquid medium (Difco) heavily from stock cultures and incubating at 23 C on a mechanical shaker (Eberbach and Sons, Co.) for 24 hr. Cells were harvested by centrifugation and washing with Hanks' solution. Heavy suspensions of yeast cells in 5 or 10 ml Hanks' solution were subsequently cooled in ice baths and subjected to homogenization or pressure chamber treatment. Solutions were then centrifuged at 10,000 g in a refrigerated centrifuge (International Co.) at 2 C for 30 min to remove cell wall debris and bacteria. The supernatant was stored at minus 15 C until used.

Yeast Dialysate

Yeast dialysate was prepared using heavy suspensions of viable S. cerevisiae in Hanks' solution. The suspension was placed in dialysis tubing (Visking Co.) and dialyzed against either sterile Hanks' solution or Karapetyan's media. Dialysis was carried out at 21 C on a mechanical shaker (Eberbach and Sons, Co.). Dialysate was assumed sterile if no bacterial or yeast growth was obtained on extract agar, nutrient broth or Sabouraud's agar after 24 hr incubation.

D. ISOLATION OF GIARDIA CULTURES

Source of Trophozoites

Giardia trophozoites were obtained from naturally infected rabbits (Rabbit Meat Co.) chinchilla (Local Chinchilla Breeders) dogs, cats, rats,

mice (Animal Care Department) and humans (Medical School Hospital). Segments of small intestine were obtained from animals, and duodenal aspirates were obtained from humans.

Isolation of Trophozoites from Specimens

Animal Specimens

Giardia trophozoites for inocula were prepared by both methods described by Karapetyan (25).

Method No. 1: A 10 to 15 cm segment of intestine was opened longitudinally and intestinal content and mucous membrane were scraped free with a scalpel. 15 to 20 ml Hanks' solution were added to this tissue and CO_2 was blown into the mixture through a small capillary pipette to fragment tissue and separate trophozoites from debris. This suspension was filtered through a washed linen cloth to eliminate remaining tissue particles. The filtrate was centrifuged at 1000 rpm for 10 min. The sediment containing trophozoites was used as inocula.

Method No. 2: Mucous membrane and intestinal content obtained as in Method No. 1, were washed with Hanks' solution. This suspension was centrifuged at 1000 rpm for 1 min to precipitate larger particles. The supernatant was recentrifuged at 1000 rpm for 10 min. The sediment containing trophozoites was used as inocula.

Human Specimens

Giardia from human duodenal aspirates were prepared by centrifuging at 1000 rpm for 10 min while still in duodenal juices. The sediment containing trophozoites was used as inocula.

Inoculation of Cultures

0.2 ml of inoculum is added to culture tubes containing 1.5 ml Karapetyan's media and 0.2 ml yeast suspension. On the second day after inoculation, 1.0 ml Karapetyan's media is added to culture tubes. Subsequently, the old fluid is removed from the culture tubes and replaced with 1.5 ml Karapetyan's media and 0.2 ml yeast suspension at daily intervals.

Inoculation of Subcultures

Stock cultures showing intensive growth were shaken vigorously in a rotational motion 5 to 10 times. All culture fluid was withdrawn. 0.5 ml of this fluid containing large numbers of trophozoites was added to culture tubes containing 1.5 ml Karapetyan's media and 0.2 ml yeast suspension. Daily media changes were continued from the first day after inoculation.

E. QUANTITATION METHOD

Microscopic Examination

Culture tubes were examined daily using one of two methods. Prior to the acquisition of an inverted microscope (Unitron), cultures were examined

under an ordinary light microscope (Bausch and Lomb) at high magnification (21 X objective and 15 X ocular) by rotating the tubes 180 degrees. With the inverted microscope it was possible to examine cultures without having to rotate them. The entire length of the growth surface of the culture tube was examined by a rapid scanning technique. Observations and recordings were taken from areas of the culture showing most intensive growth.

Ranking Method

Counting trophozoites in several hundred tubes daily proved to be impossible. It became necessary to devise a method to accurately estimate the number of trophozoites per microscopic field. A satisfactory method using a ranking system proved to be effective. The number of trophozoites per high power field (HPF) were arbitrarily assigned a rank from 0 to 6 as follows:

Rank	No. trophozoites per HPF
0	none
1	1 to 10
2	10 to 100
3	100 to 500
4	500 to 1000
5	1000 to 2000
6	2000 or greater

Rank Correlation Testing

To test the accuracy of any observer, the following protocol was used. First the observer would examine a culture tube and decide which rank was

appropriate. Immediately after assigning a rank, the actual number of trophozoites per HPF were counted by the same or a different observer, and the true rank was determined from the count. The estimated rank of the observer and the true rank were then compared using the rank correlation test. With experience it was soon possible to consistently estimate the number of trophozoites per HPF using this method.

F. MAINTAINING AEROBIC AND ANAEROBIC ENVIRONMENTS

Aerobic and Anaerobic Growth of Yeast

Aerobically cultured yeasts were obtained from Sabouraud's agar slants plugged with cotton stoppers; grown in air.

Anaerobically cultured yeasts were obtained from Sabouraud's liquid medium in tubes sealed with sterile vaseline immediately after inoculation.

Aerobic and Anaerobic Growth of Giardia

Completely aerobic conditions in Giardia cultures were obtained by plugging culture tubes with cotton stoppers and incubating in air.

Completely anaerobic conditions in Giardia cultures were maintained by placing culture tubes plugged with cotton stoppers in a phosphorus jar and igniting phosphorus prior to sealing the jar. Other anaerobic cultures were maintained in a container which was continually flushed with moistened nitrogen.

G. EXPERIMENTAL DESIGN

Cultures and Controls

Subcultures from stock rabbit and chinchilla cultures were used for both experimental and control cultures in each experiment. 6 to 10 cultures of each rabbit and chinchilla trophozoites were used for an experiment with an extra culture of both rabbit and chinchilla Giardia used for controls. Subcultures were used when the number of trophozoites in the tube had reached a rank of three or greater.

Technique for Changing to Experimental Media

The change from Karapetyan's medium to experimental media was undertaken by changing the concentrations of media in the culture tube slowly in the following daily increments.

DAY	ml KARAPETYAN'S MEDIUM	ml EXPERIMENTAL MEDIUM	TOTAL VOLUME
0	1.5	0	1.5
1	1.0	0.5	1.5
2	0.75	0.75	1.5
3	0.5	1.0	1.5
4	0	1.5	1.5

RESULTS

A. MEDIA STUDIES

Karapetyan's Media

In an effort to simplify Karapetyan's medium, the effects of single components and combinations of two and three components along with daily yeast additions on cultures of Giardia trophozoites from the rabbit and chinchilla were studied.

No single component (Figure 2) and no combination of any two components (Figure 3) would support the growth of Giardia trophozoites.

Hanks' solution, human serum and either chick embryo extract or Hottinger's digest supported the growth of Giardia (Figure 4), although the number of trophozoites obtained with either combination never reached that observed when Karapetyan's medium was used.

The combination of Hanks' solution, human serum and Hottinger's digest supported growth of Giardia at a lower level as measured by the number of trophozoites, than did Karapetyan's medium.

It was observed that subcultures from those tubes containing a combination of Hanks' solution, human serum and Hottinger's digest resulted in a slower return of the number of trophozoites to high levels than cultures receiving Karapetyan's medium.

No significant differences were observed between the growth of trophozoites from the rabbit and chinchilla.

Tissue Culture Media

The recent advancements in tissue culture techniques have given rise to many complex chemically defined media. Attempts were made to apply several of these media to the cultivation of Giardia.

Neither Medium 199 nor Eagle's Medium alone would support the growth of Giardia. In combination with human serum, Hottinger's digest, chick embryo extract and daily yeast additions, these media would support growth of trophozoites, however at lower levels than those cultures grown on Karapetyan's medium.

The use of Media NCTC 107 and 109 singly and in combination with components of Karapetyan's medium are illustrated in Figures 5 and 6. Neither media would support growth of Giardia alone, in combination with any of the components used, or with daily yeast additions. Combinations including Medium NCTC 109 consistently produced longer survival of trophozoites than did combinations including Medium NCTC 107.

No consistent difference in growth response was observed between rabbit and chinchilla trophozoites.

Toxic Effect of Frozen and Thawed Serum

During the preparation of pooled human serum, on one occasion a flask of serum inadvertently was frozen, thawed, and then refrozen. When this flask of serum was later thawed and added to cultures of Giardia in Karapetyan's medium, the number of trophozoites decreased to zero within

4 to 6 days. This phenomenon was repeated deliberately in our laboratory (30), and found to occur consistently with serum which had been frozen two or more times.

Serum Fractions

The results of substituting crystalline bovine serum albumin, salt free human serum albumin and fraction V human serum albumin for normal whole human serum are illustrated in Figure 7. Both rabbit and chinchilla trophozoites decreased in number until reaching zero within 5 days after changing to experimental media.

Animal Sera

Karapetyan's claim (25) that trophozoites grow equally well on either horse or beef serum was not substantiated by us. Horse serum substituted for human serum in Karapetyan's medium repeatedly failed to support the growth of either rabbit or chinchilla trophozoites.

No attempt was made to use beef serum as a substitute for human serum.

Rabbit serum substituted for human serum in Karapetyan's medium was also used on Giardia cultures. A rapid change from medium containing human serum to medium containing rabbit serum and the continued daily additions of this medium caused the number of trophozoites in cultures to decrease to zero in 2 to 7 days. If this change of media was conducted over a period of 4 to 6 days using the technique described in Materials and Methods, trophozoites continued to grow, but in reduced numbers as compared

to controls. No difference was observed in the response between rabbit and chinchilla trophozoites.

Peptone, Tryptone and Trypticase

Peptone, tryptone and trypticase were substituted in different concentrations for Hottinger's digest in Karapetyan's medium. Media containing either peptone or tryptone failed to support growth of trophozoites from the rabbit or chinchilla. The number of trophozoites decreased to zero in 7 to 10 days after changing to the experimental media.

Karapetyan's medium containing trypticase as a substitute for Hottinger's digest in the presence of daily yeast additions supported growth of trophozoites from both the rabbit and chinchilla. The number of trophozoites was considerably lower in cultures maintained on the trypticase medium than those maintained on Karapetyan's medium containing Hottinger's digest.

Pyruvate, Acetate, Succinic Acid and Alpha-ketoglutaric Acid

Results of substituting these compounds for glucose in Hanks' solution used on Giardia culture as Karapetyan's medium in the presence of daily yeast additions are illustrated in Figure 8. Cultures were changed from Karapetyan's medium to the experimental media using the technique described in Materials and Methods. It is readily seen that all of these compounds support growth of both rabbit and chinchilla Giardia. None of these compounds produced growth of trophozoites at levels equal to that produced by glucose. (Controls) As indicated in Figure 8, the substitution of pyruvate

and acetate produced excessive yeast growth in Giardia culture tubes, necessitating the transfer of trophozoites to clean tubes to permit continued observations.

B. YEAST AND PRODUCTS SUBSTITUTED FOR YEAST

Daily Yeast Additions

Daily additions of S. cerevisiae were essential to the growth of trophozoites. Figure 9 illustrates the effect on Giardia cultures of discontinuing yeast on the fourth day after subculture. The number of trophozoites in the cultures decreases to zero 12 to 14 days later. Figure 10 illustrates the effect on Giardia cultures after discontinuing yeast additions from the day of subculture until the sixth day. The number of trophozoites increased during the first 2 days, then decreased rapidly over the next 4 days. Renewal of daily yeast additions caused the number of trophozoites to again increase to control levels.

In all of the experiments thus far reported (Figures 2 through 10) one may observe a variable period of time before the actual effect of experimentation on Giardia cultures becomes evident. This is due to the fact that in the subculturing necessary for each experiment a variable number of yeast cells are introduced along with the Giardia. This is particularly well illustrated in Figure 10. Due to the initial increase in yeast cells in the medium, an actual increase in number of trophozoites occurs to a maximum on day 3;

following this a decline is seen over the next 4 days. Readdition of fresh yeast on day 6 was followed by a lag of 2 to 4 days before the expected increase occurred. This lag period was obliterated by adding media which was apparently toxic to trophozoites, or by adding bacteria as yeast substitutes (See Figure 11).

Bacteria

Successful culture techniques for other protozoa have included bacteria as symbionts. The results of substituting E. coli and S. faecalis for S. cerevisiae in Giardia cultures are shown in Figure 11. The rate of decrease in number of trophozoites was greater in this experiment than any observed. pH determinations revealed the culture fluid to be in a range of 5.5 to 6.0. This rapid decline in number of trophozoites has been observed in cultures receiving toxic agents such as quinacrine by Meyer and Chadd (30).

Heat Killed Yeast and Yeast Products

The results of substituting heat killed yeast, yeast autolysate and yeast extract for viable S. cerevisiae in Giardia cultures are illustrated in Figure 12. No difference was observed in the growth response between rabbit and chinchilla trophozoites. None of these substitutes for viable yeast cells would support growth of Giardia.

Yeast Homogenate and Dialysate

No homogenate obtained was completely free of intact yeast cells. Homogenates which were filtered could not be properly evaluated, as filtration removed all particles, debris, and presumably all activity for trophozoites. Cultures maintained on media containing filtered homogenates demonstrated slowly decreasing numbers of trophozoites until, at the end of 12 to 15 days, none were seen. The unfiltered homogenates with their residual viable yeast cells supported the growth of Giardia indefinitely.

A dialysate obtained from a heavy suspension of S. cerevisiae in Hanks' solution dialysed against Hanks' solution at 21 C for 24 hr currently is producing interesting results. Giardia cultures receiving Karapetyan's medium which contains the dialysate in Hanks' solution continue to grow at high levels in the absence of daily yeast additions. It should be pointed out, however, that these cultures also contain residual viable yeast cells which appear to be decreasing with time. Several such robust cultures were subcultured, a procedure which resulted in significant reduction in the number of trophozoites present in the tubes. With the addition of whole viable yeast cells, the level of Giardia is quickly restored to subcultured tubes in 1 or 2 days. With the dialysate cultures, the number of trophozoites remained depressed indefinitely. The Giardia in these tubes although decreased in number, appeared normal. The subcultures taken from these tubes, however, survived in very low numbers for only 5 to 7 days before

the number reached zero. Very few viable yeast cells were seen in either group of cultures after this subculturing procedure. An equal growth response was observed between trophozoites from the rabbit and chinchilla.

No other dialysis preparation produced comparable results on Giardia cultures.

C. AEROBIC AND ANAEROBIC GROWTH OF YEAST AND GIARDIA

Aerobic and Anaerobic Yeast Cultures

No difference was detected in Giardia cultures receiving daily additions from either aerobic or anaerobic yeast cultures.

Aerobic and Anaerobic Giardia Cultures

Cultures maintained under anaerobic conditions when placed in a phosphorus jar failed to grow. The number of trophozoites had decreased to zero by the end of the first 24 hr. Cultures maintained under anaerobic conditions in nitrogen also failed to grow, however, the number of trophozoites declined at a slower rate and the zero level was not reached until the third day.

Cultures of Giardia maintained in a standard air jacket incubator in unsealed tubes failed to grow. The number of trophozoites decreased to zero in 3 to 5 days. A determination of the pH of these culture fluids at the end of a 24 hr period revealed values from 7.6 to 8.0, suggesting the

absence of an adequate buffer.

D. ATTEMPTS TO CULTIVATE OTHER SPECIES OF GIARDIA

Animal Species

Numerous attempts to grow trophozoites from mice, rats, dogs, and cats in Karapetyan's medium in the presence of daily yeast additions failed. Numerous substitutions of tissue culture media, serum products and peptone were also unsuccessful in establishing cultures.

Human Species

On three occasions, duodenal aspirates containing Giardia trophozoites were inoculated in Karapetyan's medium. All attempts to establish an in vitro culture of Giardia trophozoites from man have resulted in the death of trophozoites ocurring in 1 to 3 days after inoculation.

DISCUSSION

A. NATURAL ENVIRONMENT OF *GIARDIA*

Symbiotic Associations *

Symbiotic associations are not new in parasitology. Thus, the successful early cultivation of trichomonads and amoebae utilized contaminating bacteria as a source of essential nutrients. More recently these protozoa have been established in axenic culture (10, 11) and it can be safely predicted that a similar pattern will follow for the various *Giardia* species.

Karapetyan (24) identified yeast-like fungi of the genera *Candida* and *Torulopsis* in frequent association with *Giardia* trophozoites. It was this suspicion of a symbiotic relationship which probably led him to include a *Candida* species in his original culture medium. During the course of examination of numerous preparations from dogs, cats, rabbits, chinchilla, mice, rats and humans, we have observed numerous slides in which yeast-like fungi were seldom detected. For this reason it seems doubtful that a symbiotic relationship is essential for *in vivo* growth of *Giardia*. Karapetyan (23) has been more impressed by the association and has actually suggested that the yeast are essential for survival of *Giardia* in the intestinal tract. Benrick (3) has unsuccessfully attempted to eliminate *Giardia* from infected mice by destroying the fungi with nystatin and amphotericin B. From his results he concluded that an effective chemotherapeutic agent for giardiasis

* For purposes of this thesis, the word symbiosis is defined as follows: "The living of two dissimilar organisms in close association or union." (Webster's New World Dictionary of the American Language, 1961).

used *in vivo* would have to act on the parasite directly, regardless of its effect on yeasts or yeast-like fungi.

There is little doubt, however, that a true symbiotic association between *Giardia* and *S. cerevisiae* exists *in vitro*. This association exhibits specificity and permanency. Results obtained in this study, suggest a symbiotic pair which is interdependent and possesses complex specific integrating mechanisms.

Our attempts to establish a similar symbiotic association between *Giardia* and species of bacteria failed. A major contributing factor may have included failure to provide an adequate buffer system capable of maintaining the optimal pH of 6.38 to 7.02 (17). Others include the specificity of *Giardia* for various enzymes, amino acids, vitamins and unknown factors.

B. YEAST AS A SYMBIONT

Killed Yeast, Yeast Extract, Autolysate and Homogenate

In microbiology yeast extracts and autolysates have frequently served to provide unusual nutrients for fastidious bacteria and protozoa. With *Giardia* however, the essential nutrient appears to be elusive and furnished only by living yeast. Heat or chemical manipulation of *S. cerevisiae* inevitably resulted in an inactive product.

The addition of numerous yeast homogenates to the medium resulted in survival but not multiplication of *Giardia*. These experiments while

encouraging, were complicated by the inevitable presence of viable yeast not destroyed by the homogenizer or the hydraulic press. Better techniques for rupturing yeast cells are reported in the literature but the apparatus is complex and costly.

Yeast Dialysate

The most encouraging yeast substitute studied to date has been the dialysate mentioned briefly in the Results (See under Yeast Products). The preparation of the dialysate has been described under Materials and Methods and is obtained from heavy suspensions of viable S. cerevisiae. Technical difficulties were encountered such as preparing large volumes of yeast cells and concentration procedures. The most serious obstacle was our inability to provide test Giardia cultures entirely free of yeast. With the dialysate excellent and prolonged growth of trophozoites was observed. In fact, the results were equal to those which we obtained when intact yeast cells were added to cultures daily. Although it is tempting to conclude that the dialysate represents the ideal yeast substitute, this cannot be done until we have observed multiplication of Giardia in a medium entirely free of viable yeast cells.

C. ATTEMPTS TO DEFINE GROWTH MEDIUM

Components of Karapetyan's Medium

From the data which have been presented it is clear that simplification of Karapetyan's medium constitutes a complex experimental problem. One of the four major components was successfully eliminated by the observation that chick embryo extract was not essential. The remaining nutrients such as human serum, Hottinger's digest and Hanks' solution were found to be necessary.

Limited attempts were made to simplify each of these components as follows: Several serum albumin fractions were tested and proved inactive as substitutes for whole serum. The toxic effect of freezing and thawing whole serum suggests that the stimulating component may be labile. In this connection Balamuth (1) has recently described a lipoprotein fraction which can substitute for whole serum in a medium used to culture amoebae.

The failure of rabbit serum to support growth of large numbers of rabbit or chinchilla trophozoites when substituted for human serum was unexpected. All sera were prepared in the same manner. Possible factors include the presence of an inhibitor in rabbit serum, the deficiency of a specific fraction present only in human serum, or the presence of a naturally occurring antibody.

Hottinger's digest and the trypticase substitute are both prepared from the enzymatic breakdown of protein and therefore, provide peptides and

amino acids. Hottinger's digest is produced from beef; the trypticase originates from casein. In the study of microbial growth in complex culture media the protein digests are eventually replaced by known amino acids. It seems likely this will also occur with Giardia but, at present, the trypticase and the prepared amino acid mixtures have provided some but not all of the properties of Hottinger's digest.

Chick embryo extract has been successfully replaced in tissue culture media with ultrafiltrates of both egg and embryo extracts (39). Meyer and Chadd (30) have successfully used an ultrafiltrate of whole egg as a substitute for chick embryo extract in Kerapetyan's medium.

Carbon sources other than glucose supported the growth of Giardia in less than maximal numbers. It was anticipated these compounds would successfully substitute for glucose because of the fact that all are metabolized by S. cerevisiae. Membrane permeability, inability to provide effective concentration and appropriate molecular structure are factors which may have accounted for the lower number of Giardia trophozoites obtained in cultures.

Tissue Culture Media

The differences in content of Medium NCTC 107 and 109 are given in TABLE VII. The variation in growth response of Giardia in these two media therefore must be explained by one or more of these three differences. Vitamin B_{12} is present in NCTC 109 and absent from NCTC 107. Yeasts are

a source of many vitamins, however, they are deficient in vitamin B_{12} as well as vitamin C, E, and K. The fact that no growth of Giardia occurred in either NCTC 107 or NCTC 109 with or without yeast additions, makes the simple deletion of vitamin B_{12} appear insignificant. NCTC 109, which contains adequate B_{12}, did not support growth of Giardia, nor did the additions of daily yeast improve the results. It must be assumed that there are other factors which are potentially growth-inhibiting in, or else are absent from these media.

The removal of three fatty acids and cholesterol from NCTC 107 and the subsequent increased growth response of Giardia to NCTC 109 raise several questions. Is the difference in growth response between these two media based on some inhibitory action resulting from the presence of the three fatty acids and cholesterol? Is there possibly a combination of the effects of adding vitamin B_{12} and the removal of the fatty acids and cholesterol? In addition, could the presence of potential growth-inhibition in both of these media be on the basis of the complex interaction of amino acids or other factors?

Even more complex is the attempted substitution of Medium NCTC 107 and 109 for Hanks' solution as shown in Figures 5 and 6. The results indicate quite clearly that the additional nutrients provided by NCTC 107 and 109 are not beneficial. In each case a medium consisting of either human serum, NCTC 107 or 109, chick embryo extract, Hottinger's digest and daily yeast allowed survival of Giardia for a maximum of only 15 days.

The controls containing Hanks' solution grew indefinitely. The answers to the above questions and the explanation of these data cannot be given on the basis of the results of this study, and must be arrived at through further investigation.

D. AEROBIC AND ANAEROBIC CULTURES

Yeast Cultures

On the basis of these data, it is impossible to arrive at definite conclusions regarding the ability of Giardia cultures to grow in the presence of yeast cells grown under anaerobic conditions. It is unlikely that yeast cells grown anaerobically and placed in stoppered culture tubes with Giardia continued to metabolize via the anaerobic pathways.

Giardia Cultures

Giardia cultures grown anaerobically in phosphorus jars were undoubtedly influenced by the toxic oxides liberated into the jar from the burning phosphorus. The data would indicate that Giardia were exposed to a toxic substance similar to that seen in the experiments with bacteria (Figure 11).

Giardia cultures grown anaerobically in nitrogen failed to survive. Reasons for this failure are unclear, however, possible factors include failure of yeast cells to provide proper nutrient or nutrients, and the inability of Giardia to metabolize under anaerobic conditions.

E. OTHER GIARDIA SPECIES

Animal Species

The repeated failure to cultivate trophozoites from the mouse and rat regardless of the type of media and substituted components remains a mystery. The consistently observed death of trophozoites in 2 to 3 days suggests an inhibition by some substance in the media. Further study using varying concentrations of amino acids, vitamins, purines, pyrimidines and other factors may be necessary for the solution of this problem.

Human Giardia

The greatest obstacle to the cultivation of Giardia from humans has been the availability of trophozoites. To adequately test Karapetyan's medium and other media, an adequate source of trophozoites is essential. In this study, three duodenal aspirates were obtained from patients and Giardia trophozoites were placed in Karapetyan's medium. Death of the trophozoites occurred in 3 days. In two of these attempts, the trophozoites had been removed from the duodenum some 30 to 60 minutes before they were placed in culture tubes, and it was noted that they were less motile than those trophozoites taken from animals and prepared for immediate culture.

SUMMARY

The *in vitro* growth requirements of *Giardia* trophozoites from the rabbit and chinchilla were studied, with the following results:

1. The ability of the 15 possible combinations of components of Karapetyan's medium to support *Giardia* growth was tested. These experiments revealed that Hanks' solution, human serum and either Hottinger's digest or chick embryo extract were necessary for growth of trophozoites.

2. Neither horse serum nor any of the serum albumin fractions tested would substitute for whole human serum in Karapetyan's medium. Rabbit serum would successfully substitute for human serum; however, decreased numbers of trophozoites resulted.

3. Of several peptones tested as substitutes for Hottinger's digest, trypticase alone proved to be successful and again, a decreased number of trophozoites resulted.

4. Several complex chemically defined tissue culture media failed to substitute for Karapetyan's medium and resultant data suggested the presence of a growth inhibition factor in these media.

5. Frequent additions of viable *S. cerevisiae* were shown to be essential to the *in vitro* growth of *Giardia* trophozoites. Of several yeast products tested as substitutes for viable yeast, a yeast dialysate appeared to be the most promising; the failure to obtain a test *Giardia* culture which was free of viable yeast cells precluded any conclusions.

6. Giardia cultures in Karapetyan's medium containing viable yeast failed to grow under either completely aerobic or anaerobic conditions.

7. Repeated attempts were unsuccessful in establishing in vitro cultures of Giardia trophozoites from the dog, cat, mouse, rat or human in Karapetyan's or other media containing viable S. cerevisiae.

8. No specific differences in the in vitro growth requirements of trophozoites from the rabbit and chinchilla were detected.

TABLE I

Preparation of Hanks' Balanced Salt Solution

1. Solution A (20 X concentration)

NaCl	160 g
KCl	8 g
$MgSO_4 \cdot 7 H_2O$	2 g
$MgCl_2 \cdot 6 H_2O$	2 g

Dissolve the above in 800 ml double distilled water.

$CaCl_2$	2.8 g

Dissolve in 100 ml double distilled water, then slowly add to above solution while mixing vigorously. Bring to final volume of 1000 ml with double distilled water.

2. Solution B (20 X concentration)

$Na_2HPO_4 \cdot 7 H_2O$	1.8 g
KH_2PO_4	1.2 g
Dextrose	20.0 g
Phenol red (0.5% solution)	80.0 ml

Dissolve the above in 1000 ml double distilled water.

3. Sterilize by either autoclaving at 110 C and 10 psi for 10 min or filtering through Seitz filter.

4. Freeze until used.

5. To make 120 ml Hanks' solution, add 6 ml Solution A and 6 ml Solution B to 120 ml sterile distilled water. Add 1 ml 7.5% sodium bicarbonate solution to 120 ml Hanks' solution.

TABLE II

Preparation of Chick Embryo Extract

12 day old embryos provide the best yield of extract.

1. Wipe the egg with 70% alcohol.
2. Crack and remove the blunt end of the egg over the air space with a sterile blunt instrument.
3. Remove the membrane with a pair of sterile forceps.
4. Remove the embryo gently by grasping neck with forceps.
5. Wash embryo with sterile Hanks' solution two times, then add equal volume of sterile Hanks' to embryos in sterile Waring blendor.
6. Blend for 1 to 2 minutes.
7. Pour into a sterile flask containing a sterile covered magnetic stirring bar and stir at refrigerator temperature for 30 min.
8. Centrifuge for 30 min at 2500 rpm.
9. Carefully remove the supernatant and dispense into 12 ml aliquots in cotton stoppered centrifuge tubes.
10. Store frozen until used.

TABLE III

Preparation of Hottinger's Digest

1. Boil 3 lb. lean beef cut into 2 or 3 in cubes in 3 liters tap water for 30 min.

2. Grind the cooked beef with a meat grinder.

3. Adjust the pH of the juice to 8.0 to 8.5 by slowly adding 40% sodium hydroxide and testing the pH at frequent intervals.

4. Add the ground beef to the juice and allow to cool to 40 to 45 C.

5. Add 6.6 g of 1:250 trypsin (Difco).

6. Pour through a large funnel into a 4 liter flask.

7. After adding 65 ml chloroform, stopper with cotton and allow to stand overnight at room temperature. Shake flask occasionally.

8. Incubate in 37 C incubator for 2 days with occasional mixing. At the end of two days, the meat should be gray in color, and the supernatant should be a straw yellow.

9. After filtering the supernatant into a container, dispense in 25 ml aliquots in screw top 50 ml bottles. Discard the sediment.

10. After autoclaving loosely capped bottles at 121 C and 15 psi for 15 min, allow bottles to cool slowly in the autoclave, then tighten screw caps and store at refrigerator temperature until used.

TABLE IV

Components of Minimal Medium Eagle (12)

INGREDIENTS	PER LITER
L-Arginine	105 mg
L-Cystine	24 mg
L-Histidine	31 mg
L-Isoleucine	52 mg
L-Leucine	52 mg
L-Lysine	58 mg
L-Methionine	15 mg
L-Phenylalanine	32 mg
L-Threonine	48 mg
L-Tryptophan	10 mg
L-Tyrosine	36 mg
L-Valine	46 mg
Choline Chloride	1 mg
Folic Acid	1 mg
Inositol	2 mg
Nicotinamide	1 mg
Pantothenate	1 mg
Pyridoxal	1 mg
Riboflavin	0.1 mg
Thiamine	1 mg
Dextrose	1.0 g
Sodium Chloride	6.8 g
Calcium Chloride	200 mg
Potassium Chloride	400 mg
Magnesium Chloride	200 mg
Monosodium Phosphate	150 mg
Sodium Bicarbonate	2.0 g
Double Distilled Water	1000 ml

TABLE V

COMPONENTS OF MEDIUM 199 (33)

Ingredients per liter			
L-Arginine	70 mg	Folic Acid	0.01 mg
L-Histidine	20 mg	Choline	0.5 mg
L-Lysine	70 mg	Inositol	0.05 mg
L-Tyrosine	40 mg	p-Aminobenzoic Acid	0.05 mg
DL-Tryptophan	20 mg	Vitamin A	0.1 mg
DL-Phenylalanine	50 mg	Calciferol	0.1 mg
L-Cystine	20 mg	Menadione	0.01 mg
DL-Methionine	30 mg	a-Tocopherol Phosphate	0.01 mg
DL-Serine	50 mg	Ascorbic Acid	0.05 mg
DL-Threonine	60 mg	Glutathione	0.05 mg
DL-Leucine	120 mg	Cholesterol	0.2 mg
DL-Isoleucine	40 mg	L-Glutamine	100 mg
DL-Valine	50 mg	Adenosinetriphosphate	1 mg
DL-Glutamic Acid	150 mg	Adenylic Acid	0.2 mg
DL-Aspartic Acid	60 mg	Ribose	0.5 mg
DL-Alanine	50 mg	Desoxyribose	0.5 mg
L-Proline	40 mg	Bacto-Dextrose	1 g
L-Hydroxyproline	10 mg	Tween 80	5.0 mg
Glycine	50 mg	Sodium Acetate	50 mg
L-Cysteine	0.1 mg	Iron (as Ferric Nitrate)	0.1 mg
Adenine	10 mg	Sodium Chloride	8 g
Guanine	0.3 mg	Potassium Chloride	0.4 g
Xanthine	0.3 mg	Calcium Chloride	0.14 g
Hypoxanthine	0.3 mg	Magnesium Sulfate	0.2 g
Thymine	0.3 mg	Disodium Phosphate	0.06 g
Uracil	0.3 mg	Monopotassium Phosphate	0.06 g
Thiamine	0.01 mg	Sodium Bicarbonate	0.35 g
Riboflavin	0.01 mg	Bacto-Phenol Red	0.02 g
Pyridoxine	0.025 mg	Carbon Dioxide	to pH 7.2
Pyridoxal	0.025 mg	Triple Distilled Water	1000 ml
Niacin	0.025 mg		
Niacinamide	0.025 mg		
Pantothenate	0.01 mg		
Biotin	0.01 mg		

TABLE VI

COMPONENTS OF MEDIUM NCTC 107 (13)

	mg/100 ml
L-Alanine	3.148
L-Aminobutyric Acid	0.551
L-Arginine	2.576
L-Aspartic Acid	0.991
L-Asparagine	0.809
L-Cystine	1.049
D-Glucosamine	0.320
L-Glutamic Acid	0.826
L-Glutamine	13.573
Glycine	1.351
L-Histidine	1.973
Hydroxy-L-Proline	0.409
L-Isoleucine	1.804
L-Leucine	2.044
L-Lysine	3.075
L-Methionine	0.444
L-Ornithine	0.738
L-Phenylalanine	1.658
L-Proline	0.613
L-Serine	1.075
L-Taurine	0.418
L-Threonine	1.893
L-Tryptophan	1.750
L-Tyrosine	1.644
L-Veline	2.500
Thiamine Hydrochloride	0.0025
Riboflavin	0.0025
Pyridoxine Hydrochloride	0.00625
Pyridoxal Hydrochloride	0.00625
Niacin	0.00625
Niacinamide	0.00625
Pantothenate, Calcium Salt Dextrorotatory	0.0025
Biotin	0.0025
Folic Acid	0.0025
Choline Chloride	0.125
i-Inositol	0.0125
p-Aminobenzoic Acid	0.0125
Vitamin A	0.025
Calciferol	0.025
Menadione	0.0025
a-Tocopherol Phosphate Disodium Salt	0.0025
Glutathione-Monosodium	1.01
Ascorbic Acid	4.99
Cysteine Hydrochloride	25.99
Diphosphopyridine Nucleotide	0.70
Triphosphopyridine Nucleotide-Sodium Salt	0.10
Coenzyme A	0.25
Cocarboxylase	0.10
Flavin Adenine Dinucleotide	0.10
Uridine Triphosphate Disodium Salt	0.10
Deoxyadenosine	1.00
Deoxycytidine-HCl	1.00
Deoxyguanosine	1.00
Thymidine	1.00
5-Methylcytosine	0.01
Methyl Linoleate	0.10
Methyl Linolenate	0.10
Methyl Arachidonate	0.10
Cholesterol	0.20
Tween 80	2.25
Glucuronolactone	0.18
Sodium Glucuronate	0.18
Sodium Acetate	5.0
Phenol Red	2.0
Sodium Chloride	680.0
Potassium Chloride	40.0
Calcium Chloride	20.0
Magnesium Sulfate	20.0
Sodium Monobasic Phosphate	14.0
Sodium Bicarbonate	220.0
Dextrose	100.0

TABLE VII

Components of Medium NCTC 109 (35)

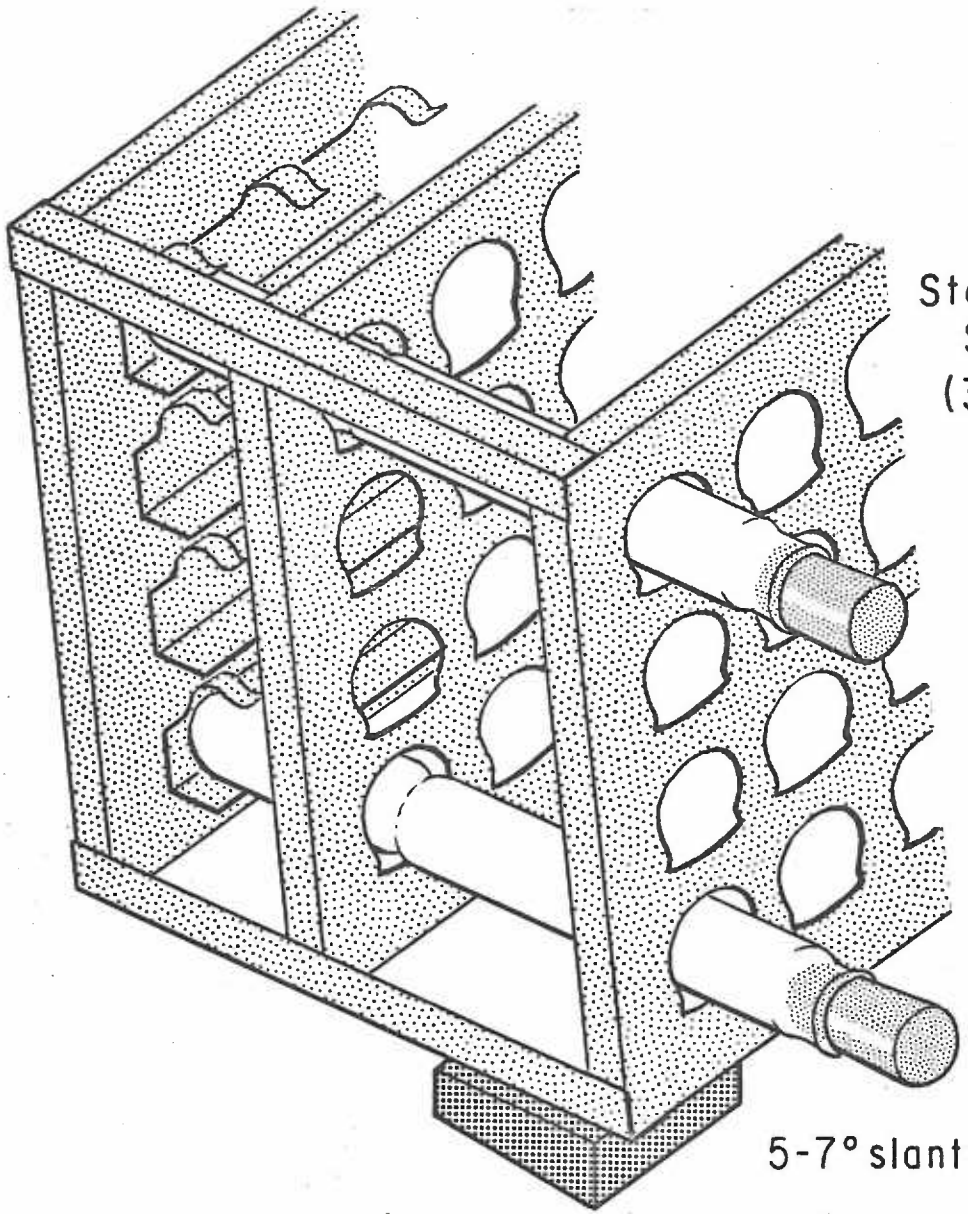
Medium NCTC 109 is identical with NCTC 107 except for the following changes:

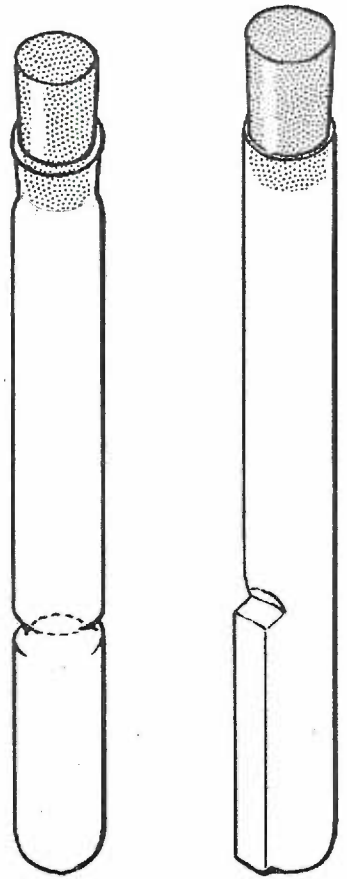
1. Vitamin B_{12} is added at a concentration of 0.01 mg/ml.
2. Methyl linoleate, methyl linolenate, methyl arachidonate and cholesterol are omitted.
3. The amount of Tween 80 is reduced to 1.25 mg/100 ml.

FIGURE 1

CULTURE APPARATUS

Stainless steel culture tube rack slanted at 6 degrees. Constricted and Leighton type tissue culture tubes with rubber stoppers.





Constricted Leighton
Tissue Culture Tubes

FIGURE 2

Growth of Giardia trophozoites on single components of Karapetyan's medium.

GROWTH OF GIARDIA TROPHOZOITES ON SINGLE COMPONENTS OF COMPLETE GROWTH MEDIA

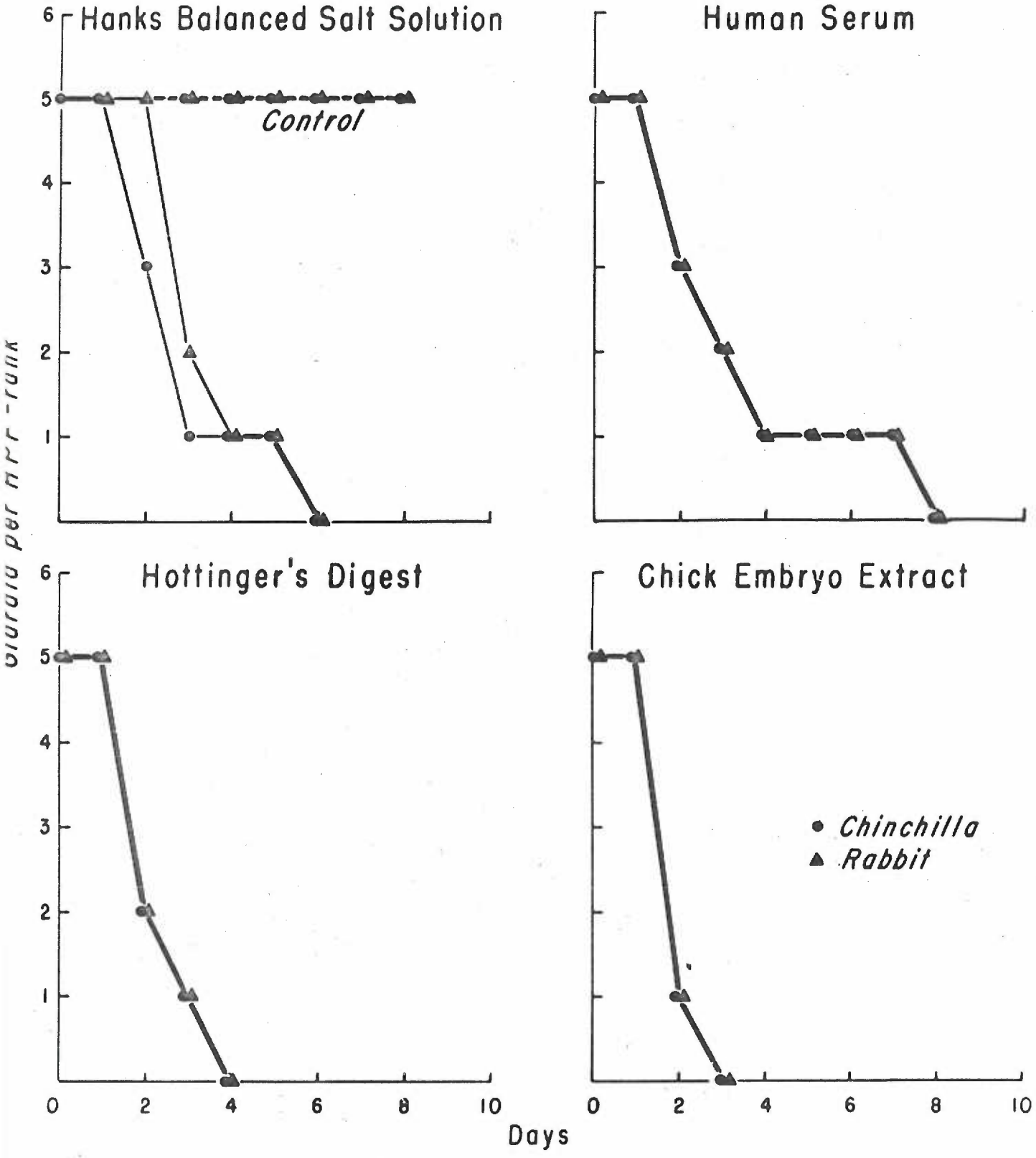


FIGURE 3

Growth of Giardia trophozoites on combinations of any two components of Karapetyan's medium.

GROWTH OF GIARDIA TROPHOZOITES ON ANY TWO COMPONENTS OF COMPLETE GROWTH MEDIA

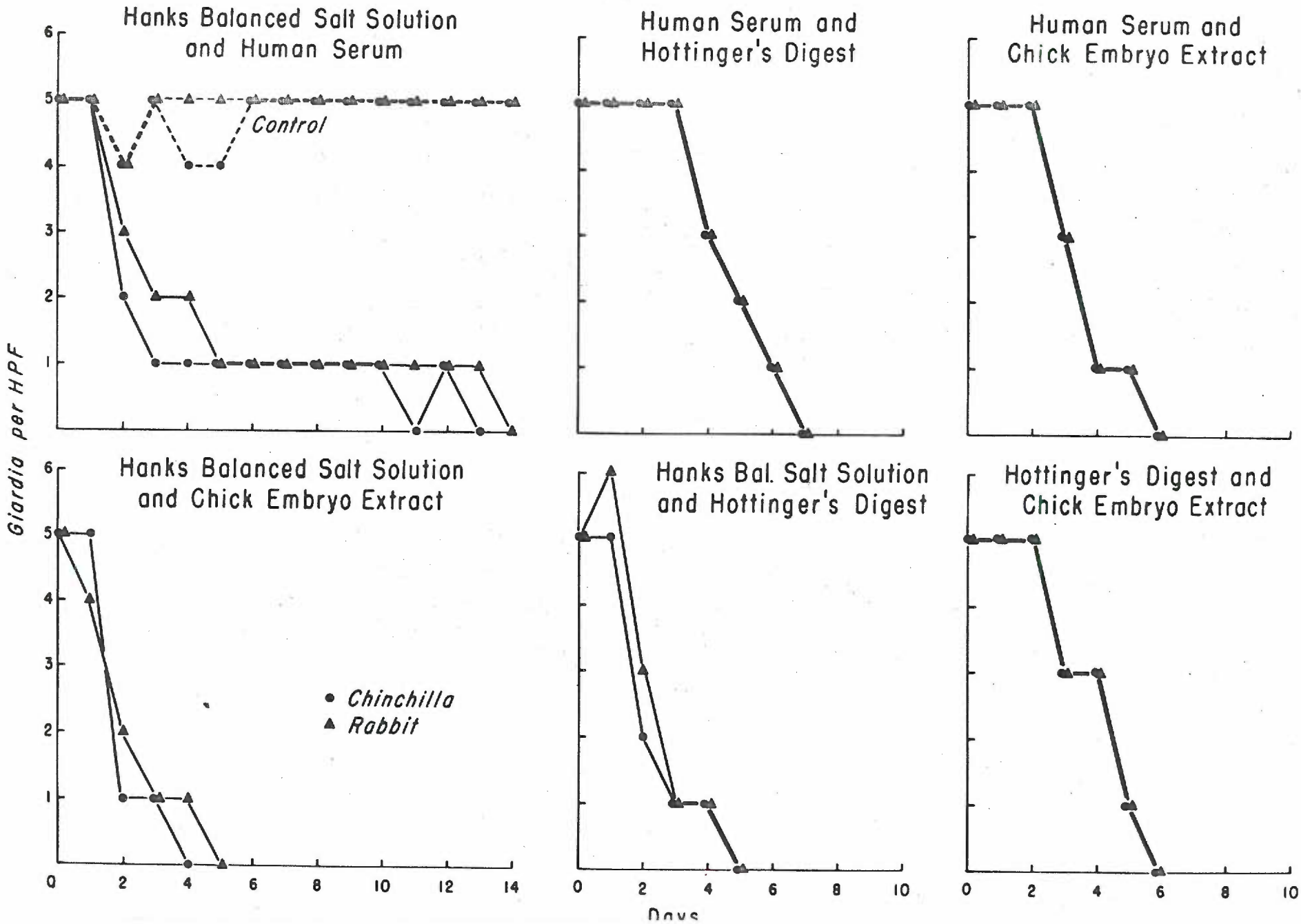


FIGURE 4

Growth of Giardia trophozoites on any three components of Karapetyan's medium.

GROWTH OF GIARDIA TROPHOZOITES ON ANY THREE COMPONENTS OF COMPLETE GROWTH MEDIA

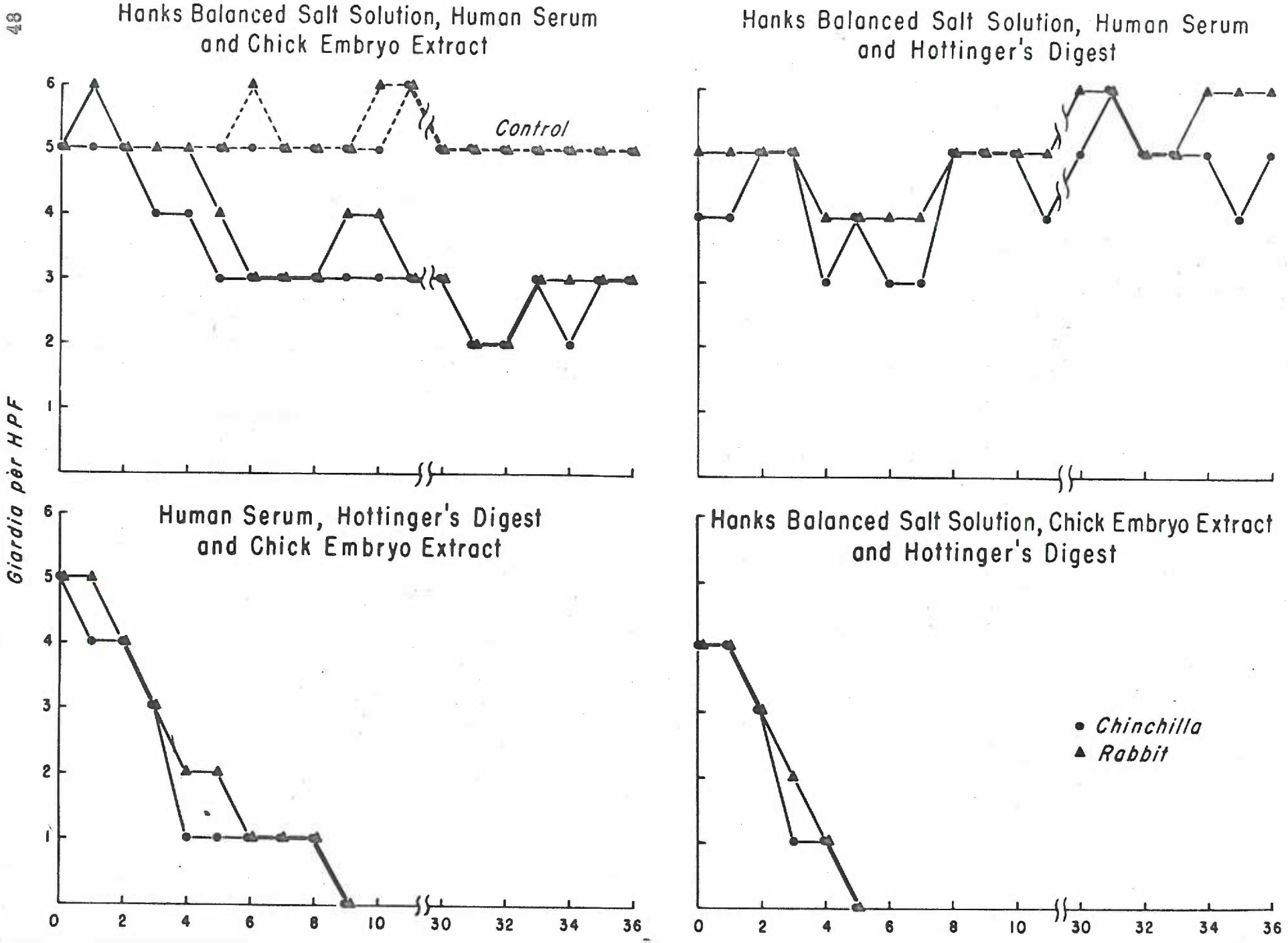


FIGURE 5

The effects of Medium NCTC 107 and added nutrient components on Giardia trophozoites.

EFFECTS OF MEDIUM NCTC 107 AND ADDED NUTRIENT COMPOUNDS

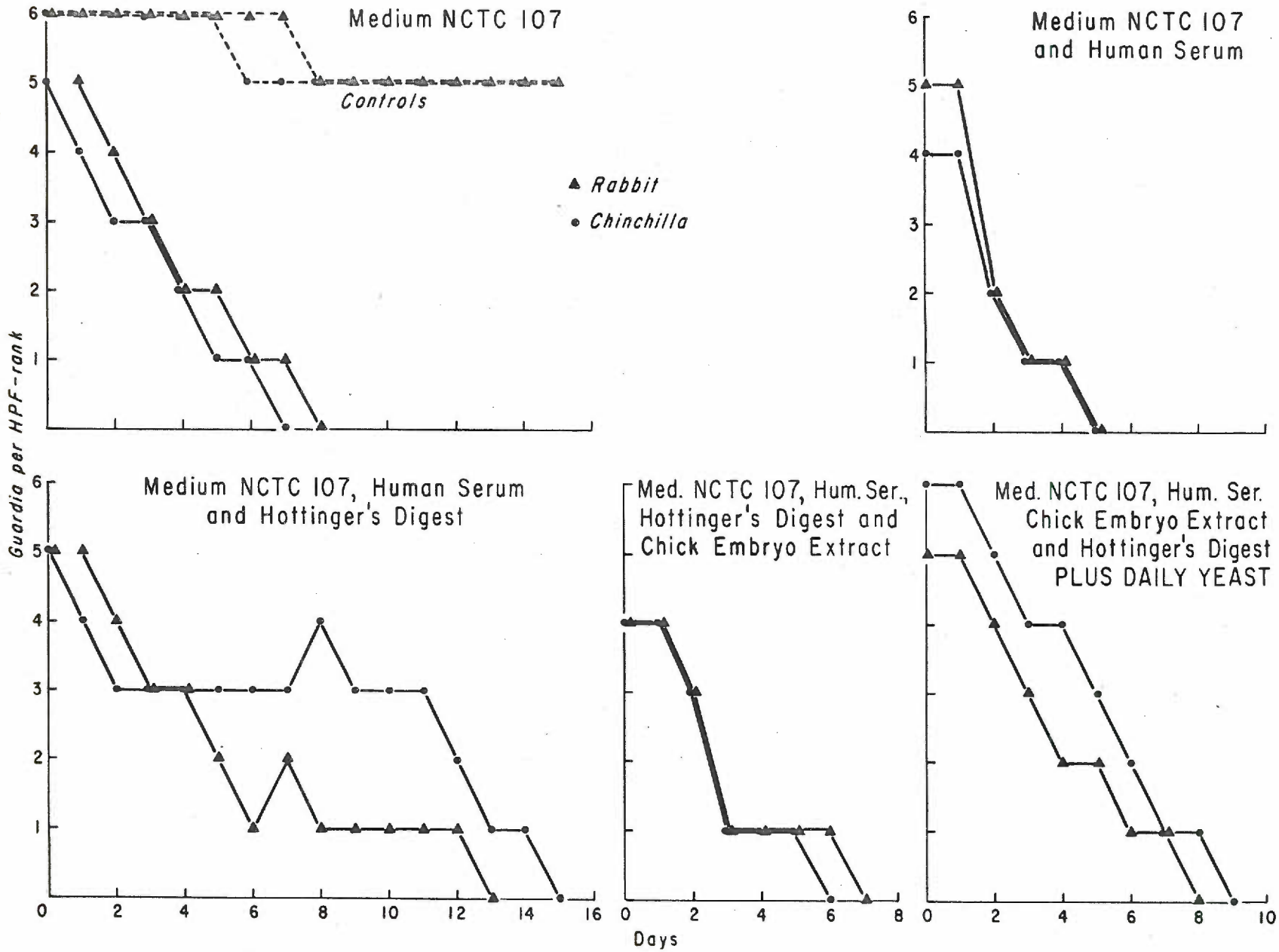


FIGURE 6

The effects of Medium NCTC 109 and added nutrient components on *Giardia* cultures.

EFFECTS OF MEDIUM NCTC 109 AND ADDED NUTRIENT COMPOUNDS

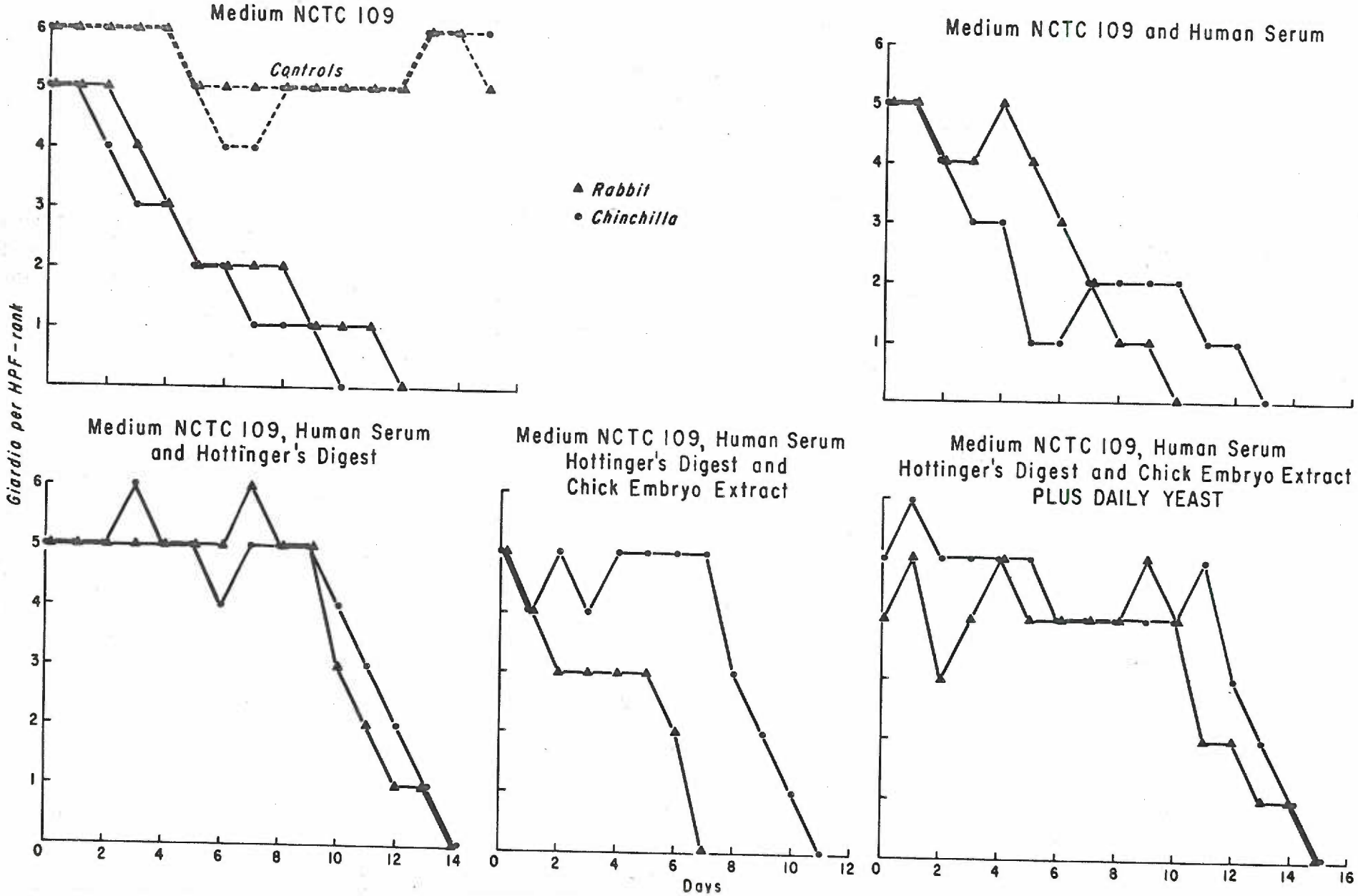


FIGURE 7

Giardia growth in Karapetyan's medium containing serum products substituted for whole human serum.

SERUM PRODUCTS SUBSTITUTED FOR HUMAN SERUM IN GROWTH MEDIUM

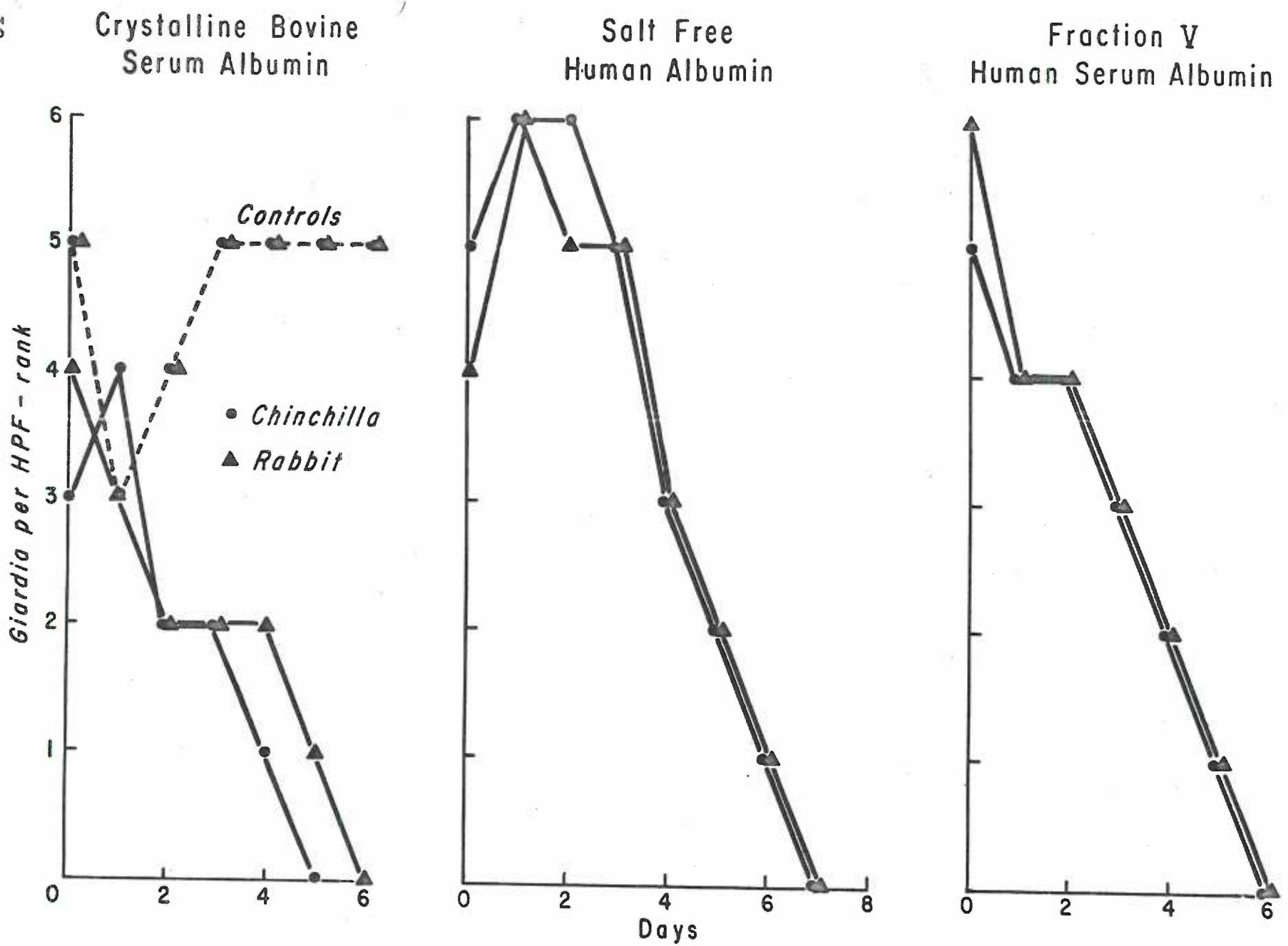


FIGURE 8

Effects of substituting acetate, pyruvate, succinic acid and alpha-ketoglutaric acid for glucose in Karapetyan's medium containing viable yeasts and Giardia trophozoites.

EFFECTS OF ALTERNATE CARBON SOURCES AS GLUCOSE SUBSTITUTES

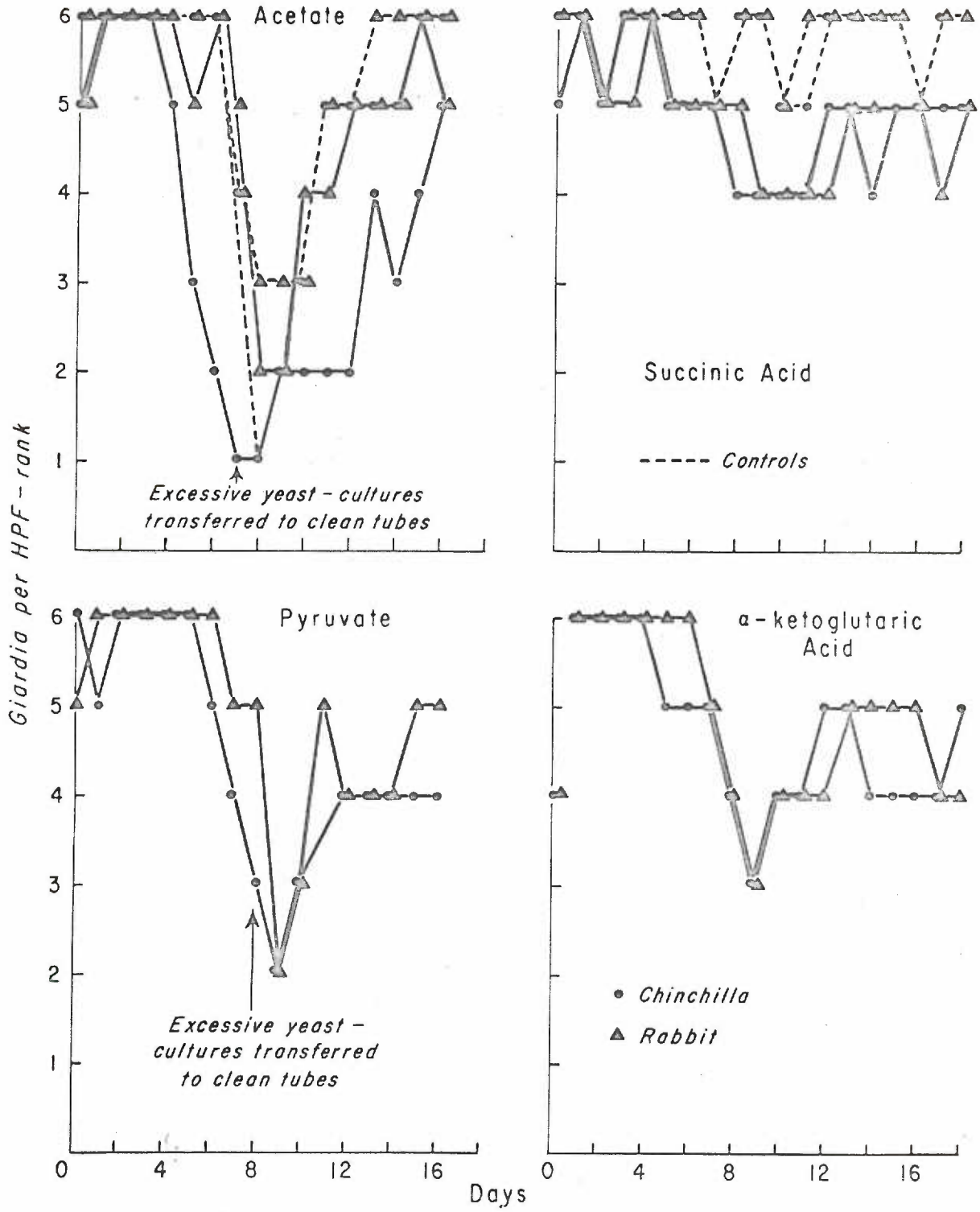


FIGURE 9

Effects of discontinuing yeast additions to Giardia cultures on the fourth day after subculture.

EFFECTS OF DISCONTINUING YEAST AFTER FOURTH DAY

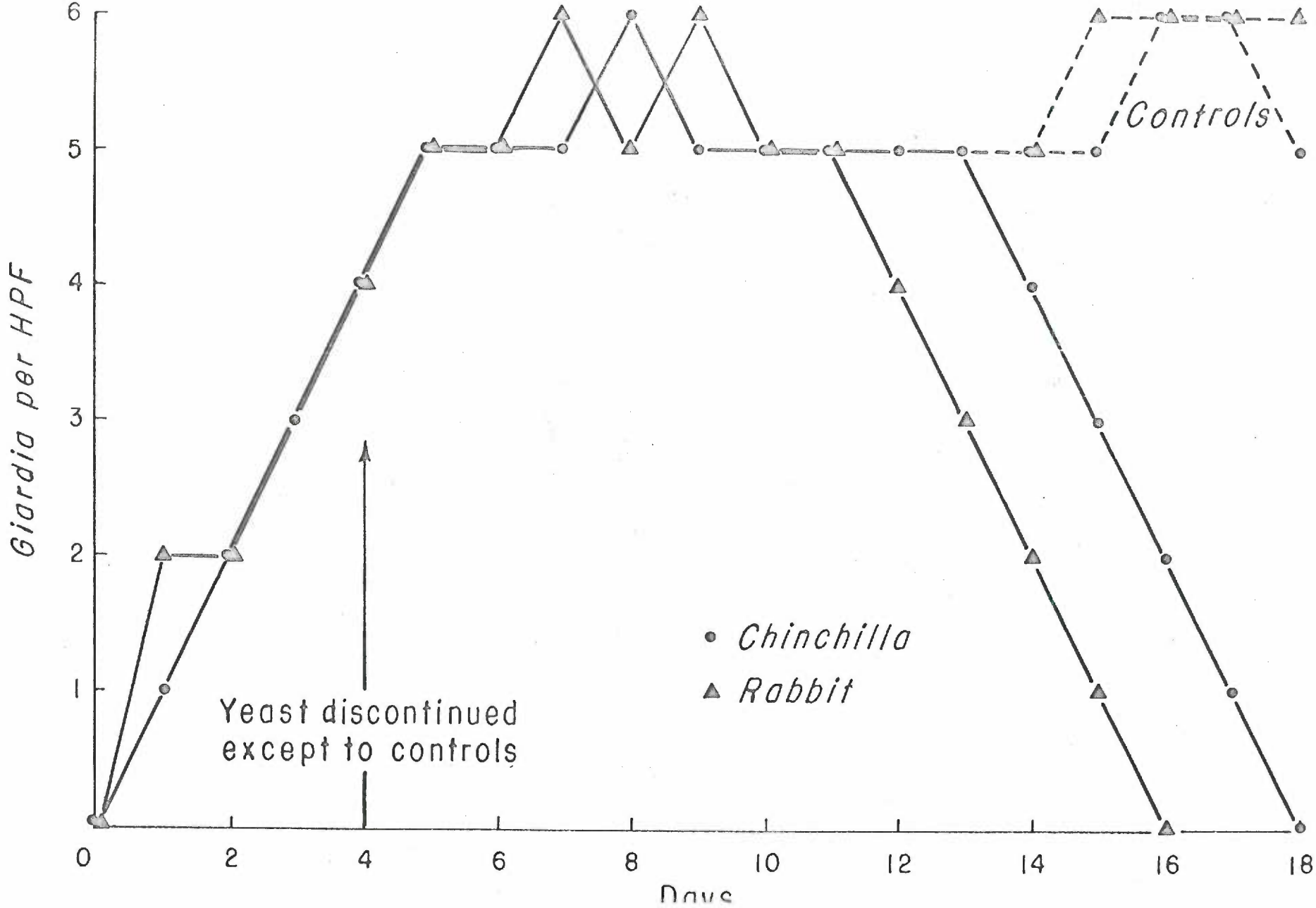


FIGURE 10

Effects of discontinuing daily yeast additions to Giardia cultures until the sixth day after subculture.

EFFECTS OF DISCONTINUING DAILY YEAST UNTIL SIXTH DAY

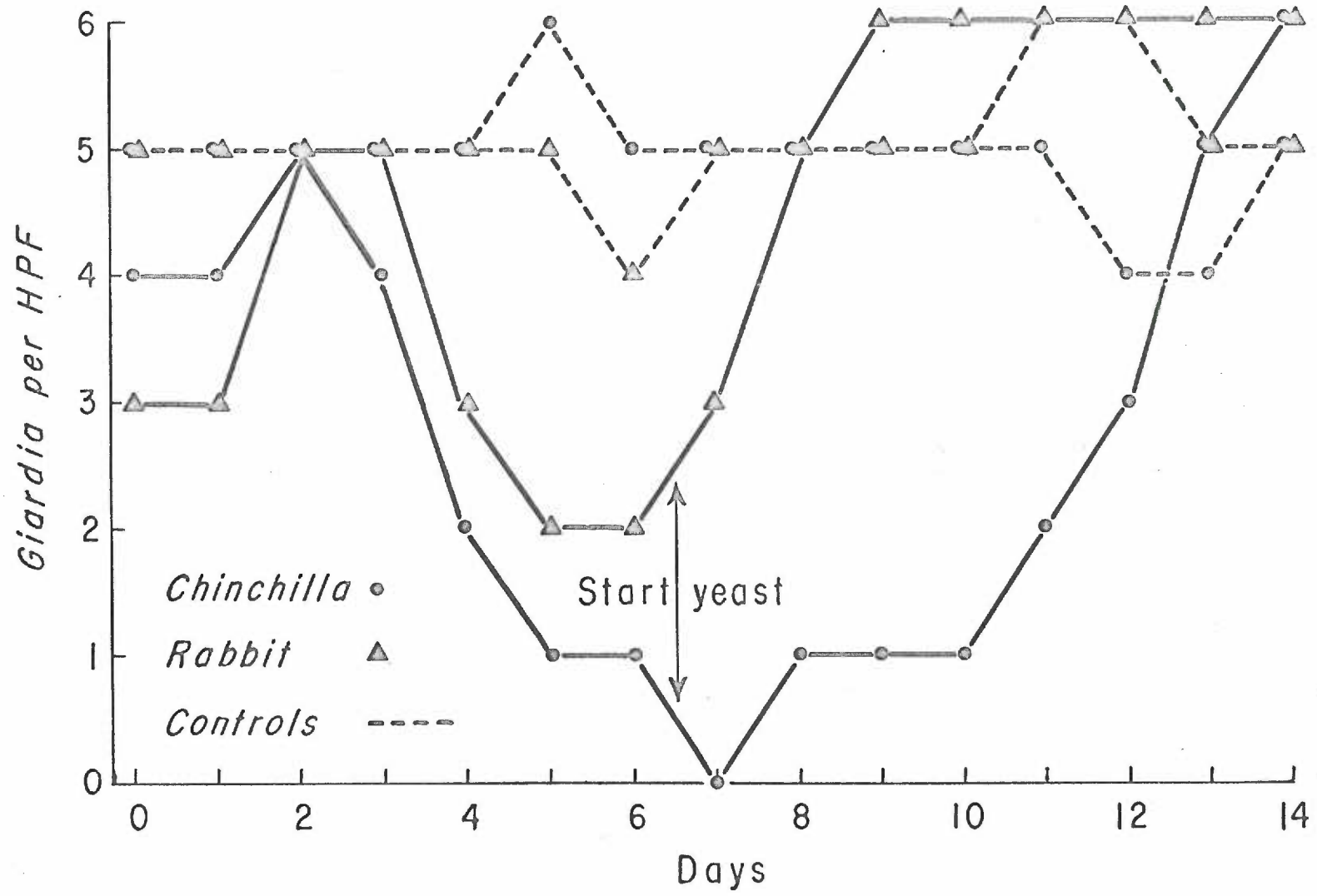


FIGURE 11

Effect on Giardia cultures receiving daily additions of bacteria as substitutes for yeast.

EFFECTS OF BACTERIA SUBSTITUTED FOR YEAST

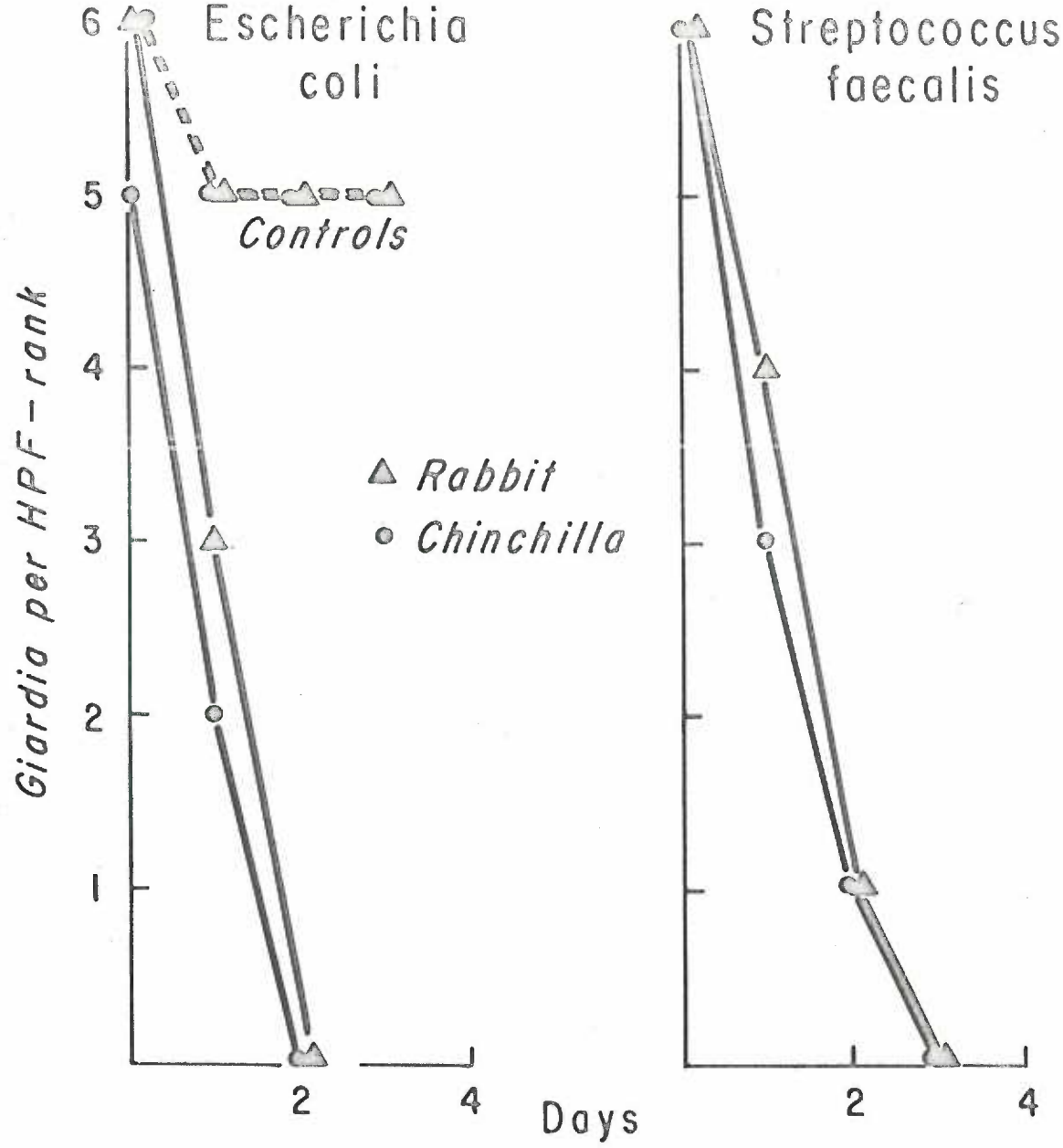
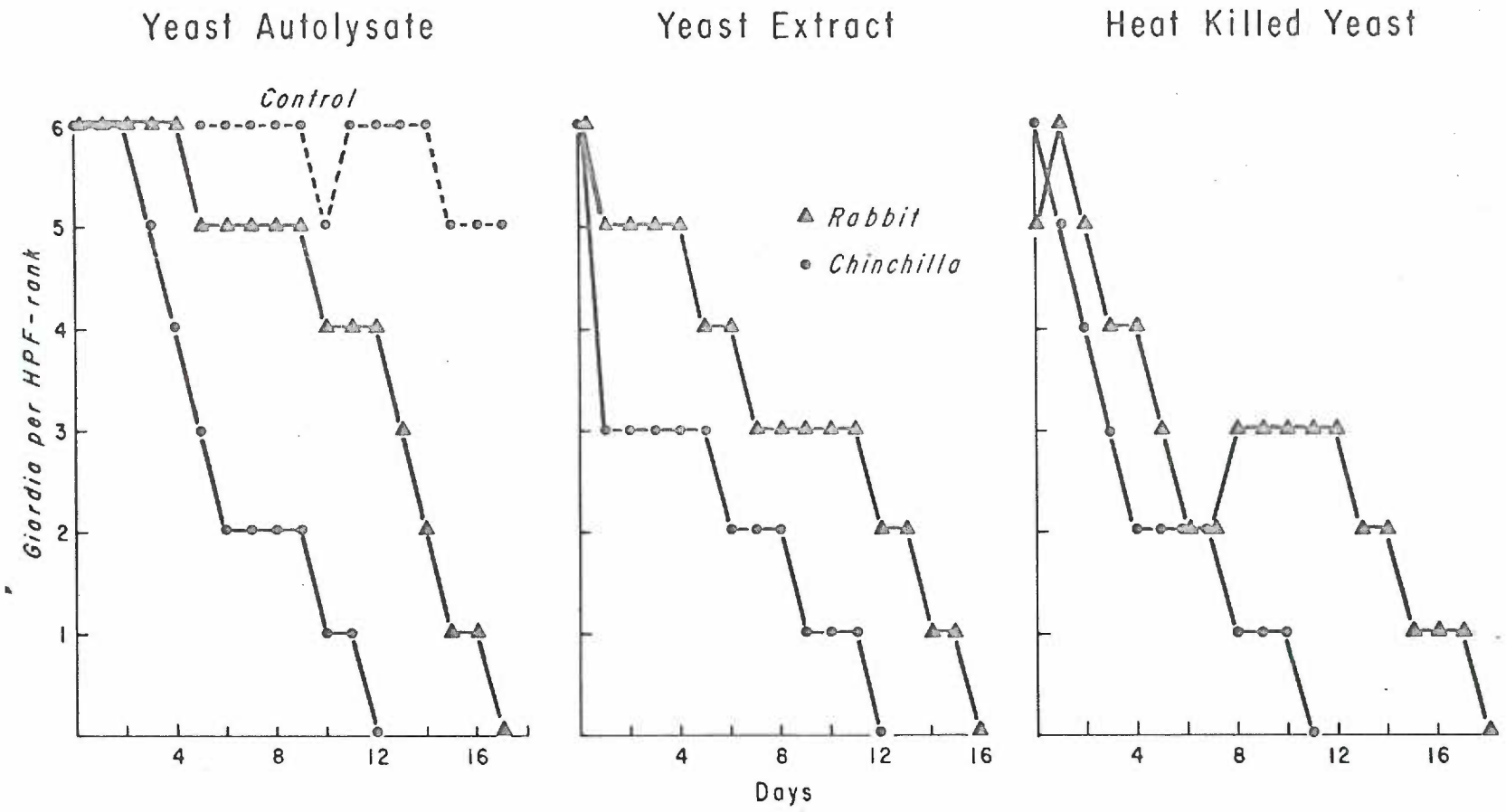


FIGURE 12

Effects of daily additions of yeast autolysate, yeast extract and heat killed yeast to *Giardia* cultures as substitutes for viable yeast.

EFFECTS OF KILLED YEAST AND YEAST PRODUCTS SUBSTITUTED FOR VIABLE YEAST



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