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REPLACEMENT OF WHOLE SERUM FOR THE *IN VITRO* CULTIVATION OF *TRICHOMONAS FOETUS*

By

Jack Jenks Stockton

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1962

Doctoral Committee

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PREFACE

Just as no individual is an island unto himself no bit of research is strictly the product of the thought, imagination, skill and industry of the doer. In the final analysis contributions of many impinge on any given research project.

Sincere and humble appreciation is expressed to Professor Richard J. Porter for his unfailing and constant encouragement. His discerning advice and critical analysis are also gratefully acknowledged. To the other members of the Doctoral Committee acknowledgement and thanks are given for their time, interest and understanding.

Many people have contributed indirectly along the way. Not the least of these has been my family. To them a special recognition is due. Smiling when they might have cried, understanding when they might have questioned, encouraging when they might have discouraged and inspiring when they might have ridiculed they stand as a constant source of pride and motivation.

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INTRODUCTION

One of the physiological characteristics of many of the parasitic flagellate protozoans is the requirement of blood or blood serum for *in vitro* cultivation. This need of whole blood or animal serum, or of some component of these substances, for propagation outside of a living animal is not unique with these protozoa but is similarly observed with a number of bacteria. Likewise for the successful propagation of cell cultures in which viruses and rickettsiae may be grown, animal serum is a necessary constituent of the nourishing substrate. Whole blood and serum are biologically and chemically complex substances and contain representative carbohydrates, fats, and proteins in addition to substances classified as enzymes and vitamins.

That whole blood, or serum, is generally recognized as an excellent enrichment for the *in vitro* cultivation of a number of so called fastidious microorganisms would indicate that these substances are a good source of life-sustaining elements. Owing to the aforementioned complexity, it would not be surprising to learn when the factors necessary for subsistence and multiplication in a given instance are delineated that a mosaic of substances contribute rather than a single component.

Of the protozoan organisms, many of the class Flagellata have been successfully cultivated in the laboratory. In this group certain species in the order Trichomonadida have been especially well studied from the standpoint of optimum culture environment, and a few have been studied to determine more precise metabolic activity. Those which have received the most attention are the pathogens Trichomonas vaginalis Donné 1836 , Trichomonas foetus Riedmüller 1928 and Trichomonas columbae Rivolta 1878 (=gallinae). Of these, the first has been most thoroughly studied probably because of its importance as a parasite of man. Of the two parasites of other animals T. foetus has received more attention undoubtedly because of its economic significance in relation to impaired breeding and abortions which it causes in cattle. From the standpoint of effect on the host, T. gallinae is the most pathogenic of the three, chickens, pigeons and doves often succumbing to infection with this organism. These flagellates are commonly carried bacteria-free in artificial culture media in the laboratory. Many media have been devised and recommended for their in vitro propagation. These have varied in composition from the highly complex to the comparatively simple. One thing which has been common to all media which support the growth

of these trichomonads in sustained serial subculture is the inclusion of whole blood or serum or, the inclusion of tissue fragments, especially liver. Routinely, it has been impossible to maintain cultures without this form of enrichment. It is thus evident that some substance or substances present in serum are needed by these organisms to allow them to metabolize and reproduce.

Limited attempts have been made to isolate and characterize the components of serum which are essential for supporting maximum growth of the trichomonads *in vitro*. Of the approaches which have been made, none has employed the use of serum fractions obtained by either the alcohol fractionation method or the salting-out method to learn if any particular fraction, or fractions, thus obtained might be used as a substitute for the intact serum. The use of such fractions appears to be one logical approach in an attempt to learn if, perhaps, one serum component is the major factor in supporting growth of the trichomonads or if a mosaic of components is required. A study of the ability of serum fractions, obtained by both the alcohol fractionation technique and the salting out technique, to replace whole serum in a comparatively simple medium for the cultivation of *T. foetus* constitutes the major part of

this investigation. Other methods of obtaining serum fractions were also tried in an attempt to obtain data on the characteristics of growth-supporting substances present. Limited studies were made in attempting to learn the precise nature of the fraction which could replace whole serum in the artificial culture medium.

Aside from academic interest, it was hoped that some insight into the more detailed nutritional requirements of the trichomonads might be gained.

HISTORICAL

I. TAXONOMY AND MORPHOLOGY OF THE TRICHOMONADS.

a. General. Kirby (1947) felt that Trichomonas and allied genera should be grouped in a new order Trichomonadida. Four families were described: Monocercomonadidae, Devescovinidae, Calonymphidae, and Trichomonadidae. The trichomonads are for the most part parasitic, very few free-living species having been described. Bishop (1935) described Trichomonas keilini from a pond in Lincolnshire, England, and Hollande (1939), Coelotrichomastix convergus in France. The parasitic species infect a wide variety of hosts including invertebrates (mollusks, insects, leeches) cold-blooded vertebrates (amphibians, fishes, reptiles) and warm-blooded vertebrates (birds and mammals). The relationship is often one of simple commensalism. As mentioned previously, the three species, T. vaginalis in man, T. foetus in the cow, and T. columbae in the pigeon are best known for the harmful effects they produce in the host.

Kirby (1947) described the trichomonads as follows:

"Trichomonadida Ord. Nov.

Flagellata with mastigont in known forms of three or four to six flagella, one of which is typically differentiated

as a trailing flagellum, an axostyle, and a parabasal body of the Janicki-type which originates de novo in relation to the basal granule system. There may be loss or modification of certain organelles and addition of others. Each mastigont system is originally associated with one nucleus. Mitosis is of the Grassi-type, in which an extranuclear spindle or paradesmose is present. Polymonad organization exists in one series.

"Trichomonadidae Wenyon, 1926

Trichomonadida with an undulating membrane and costa. Type genus Trichomonas Donné, 1836."

b. Trichomonas foetus. Trichomonas foetus, the trichomonad used for most of the present work, was named by Riedmüller (1928). It had previously been named Trichomonas utero-vaginalis vitulae by Mazzanti (1900). The generic name, Trichomonas, has been used by many authors and Tritrichomonas by some. Wenrich and Emmerson (1933) were responsible for the designation Tritrichomonas foetus (Riedmüller); however, Wenrich (1935 and subsequently) used the generic name Trichomonas. Morgan (1944) used the name Trichomonas foetus, but Morgan and Hawkins (1948) used Tritrichomonas. Kirby (1951) decided, on the basis of morphology, the generic name should be Tritrichomonas and

advanced arguments for the name Tritrichomonas foetus (Riedmüller, 1928) Wenrich and Emmerson, 1933. Since there is no general agreement and since a number of names have been suggested and used, it appeared to be a matter of personal choice as to the name selected. I have chosen to designate the organism Trichomonas foetus, primarily on the basis of priority.

A number of investigators have commented on the morphology of T. foetus. The following brief description is taken in part from Riedmüller (1928), in part from Morgan (1944), and in part from Kirby (1951). Much of the descriptive literature is based on observations made of wet mounts from either pure culture or infected fetal fluids by means of dark field microscopy.

The organisms vary in length from 10 to 25 μ with an average of approximately 16 μ. Their width is from 5 to 10 μ with an average of about 7 μ. They are in general pyriform in over-all outline but in the living actively motile state, they are very pliable and change shape appreciably. Older specimens, or those exposed to what might be considered adverse conditions, tend to become nearly spherical.

Three anterior flagella, all of approximately equal length and as long as, or slightly longer than the body, leave the body at a position slightly subapical. They are united in a column for a short distance from the point of emergence from the soma and cannot be distinguished. Beyond this column, each flagellum is easily seen in dark field. They appear stout and are of equal thickness for all their length. Riedmüller (1928) stated that the three flagella usually originate in a small indentation. These organelles are quite active and have a characteristic "whip-like" action. Morisita (1939), Gehring and Murray (1933), and Kirby (1951) have described the occurrence of a fourth flagellum on rare occasions.

The identifying feature of the Trichamonadidae is the presence of an undulating membrane and a costa. The membrane of T. foetus has from three to five waves and may or may not extend for the full length of the body. In larger specimens it frequently occupies not more than 2/3 or 3/4 of the length. A filament runs the length of the undulating membrane at its outer boundary and is commonly designated the marginal filament. This emerges from the undulating membrane at the posterior extremity and extends posteriorly as the posterior free flagellum. It compares in length and

thickness to the anterior flagella. The undulatory movement of the membrane does not continue in the flagellum. Instead the flagellum beats backward and forward, and in one position it extends almost straight posteriorly. The costa extends along the base of the undulating membrane to the posterior limits of the body. In dark field, it tapers toward either end and displacement of the undulating membrane is accompanied by a displacement of the costa, thus indicating a close association between the two.

An axostyle extends the length of the organism and projects a variable distance from the posterior extremity. The posterior projection is pointed, and there is no evidence that it is covered by cytoplasm. Toward its anterior end it gradually expands into a capitulum. In dark field, the margins appear in outline as bright lines. According to Kirby (1951), doubling of the axostyle is fairly frequent in culture and is not considered a part of the normal division process.

The nucleus and numerous granular and vacuolar inclusions can be seen by dark field illumination. At high magnification, the granules can be seen to be in a state of very active Brownian movement.

II. CULTURE AND NUTRITION OF THE TRICHOMONADS.

a. Culture of Trichomonads. Chatton (1918a, 1918b, 1918c) obtained a bacteria-free culture of Trichomastix colubrorum from a lizard, the gecko (Tarentola mauritanica). The first culture was obtained in blood agar (N.N.N. medium), and later it was cultured in a base containing animal organs or blood. This organism could not be maintained in agar-ascitic fluid medium, in ascitic fluid, or in ordinary broth, but the addition of fragments of some organ such as spleen or liver was necessary. Acidified nutrient broth enriched with fresh rabbit blood was excellent, but rabbit serum alone added to this same broth was ineffective. Cailleau (1936a) observed under the same conditions the complete suitability of horse serum for the multiplication of this flagellate. Since this early work, a number of species have been isolated in pure culture, some of them only with the aid of antibiotics.

Bos (1932, 1933) isolated T. columbae for the first time in bacteria-free culture. The materials for the inoculation of a diphasic medium, consisting of Locke's solution, coagulated egg and horse serum (L.E.S. medium), were heart blood or liver tissue collected from pigeons at the height of infection.

Trichomonas foetus was first obtained in pure culture by Witte (1933). The flagellates were present in pure culture in the uterine pus of a cow with pyometra. They grew well from the first inoculation into an egg medium with added blood, or in broth with added horse serum, but did best in blood broth (5-10 per cent blood), with a pH optimum of 7 to 7.6 and a temperature of 37 C. Sedlmeir (1942), Plastridge (1943), Mahmond (1944), Morgan (1946), and Williams and Plastridge (1946) reported the use of antibiotics for obtaining pure cultures of this organism from specimens containing bacteria.

Trussell (1940) was the first to report success in growing T. vaginalis in pure culture. Here, as with T. foetus, the use of antibiotics greatly facilitated growing this organism bacteria-free as reported by Adler and Pulvertaft (1944), Johnson, Trussell, and Jahn (1945) and Quisno and Foter (1946).

From the time of the early culture work on the trichomonads dating back to the early 1930's, a number of media have been suggested for growing these organisms. Many of these have been highly complex, a few comparatively simple, but all have had in common the inclusion of blood, or some blood derivative, or animal tissue, usually liver.

Riedmüller (1936) demonstrated that for T. foetus in a broth-blood medium, serum was the fraction necessary for its multiplication, while the red cells, washed or hemolyzed, were devoid of activity. He also reported that the active factor in serum was thermostable and could withstand heating to 90 C for 1 hour. Trussell (1947) reviewed the literature on the cultivation of T. vaginalis up to that time and indicated that serum or whole blood had been an essential ingredient of every successful preparation.

The original bacteria-free cultivation of T. foetus by Witte (1933) was made in a comparatively simple medium which bears his name. It consisted of:

Peptone	10.0 gm
Sodium chloride . . .	3.0 gm
Sodium phosphate . . .	3.0 gm
Distilled water . . .	1000.0 ml

After autoclaving, either sterile defibrinated blood or serum was added in a final volume of 5 to 10 per cent. Cailleau (1934) reported that most European strains grew well in this medium but that it would not maintain the Glaser (American) strain. Morgan (1944) confirmed the inability of this medium to maintain a majority of American

strains tested. Pop and Ogneru (1937), Wittfogel (1935), Senna (1936), Diernhofer (1936) and Riedmüller (1936) have reported success with the use of this medium for the serial subculture of T. foetus, all of the strains tested having their origin in Europe.

Gehring and Murray (1933) introduced the use of Locke's-Egg-Blood Medium (L.E.B.), which since has undergone many changes and modifications for use in the cultivation of T. foetus. The medium actually was a derivative of the Boeck-Drbohlav medium devised for the cultivation of Entamoeba histolytica. The original medium of Boeck and Drbohlav (1925) consisted of a slant, composed of whole eggs mixed with Locke's solution and coagulated by heat, overlaid with a mixture of sterile Locke's solution and one part of sterile, inactivated human serum. Gehring and Murray modified this by substituting 0.5 ml of fresh defibrinated rabbit blood in 100 ml of Locke's solution with the addition of 0.1 ml of a 1:1000 aqueous solution of crystal violet. Cameron (1935) was successful in the use of this medium in studies on bovine trichomoniasis and was able to maintain T. foetus for 30 days. Glaser and Coria (1935) used L.E.B. medium for their studies on purification of T. foetus from bacterially contaminated specimens.

They, however, substituted Ringer's solution for Locke's solution in the formula. Rees (1937), attempting purification of T. foetus by microisolation procedures, used L.E.B. medium but substituted Dorland's modified Ringer's solution in place of Locke's solution. Daniel (1940) used the same formula as Rees but modified the preparation procedures by autoclaving the entire mixture at 15 to 18 pounds pressure for 15 to 20 minutes after the addition of the serum -Ringer's solution overlay. Blood serum from the horse, cow or sheep gave results equally as good as rabbit serum or defibrinated rabbit blood. Andrews and Lyford (1940) further modified the basic L.E.B. medium by the use of 0.7 per cent NaCl in place of Locke's solution. They also reported the successful use of beef extract or beef infusion broth as a substitute for the saline in the saline-serum overlay.

Without question the best known, and perhaps currently the most widely used, culture medium for trichomonads is that developed by Johnson and Trussell (1943) and designated by them CPLM medium. The formula for and preparation of this medium are as follows:

Bacto peptone	32.0 gm
Bacto agar	1.6 gm
Cysteine HCl	2.4 gm
Maltose	1.6 gm
Liver infusion (Bacto)	320.0 ml
Ringer's solution (NaCl 0.6%, $NaHCO_3$, KCl & $CaCl_2$, 0.01% each).	960.0 ml
NaOH (1N)	11-13.0 ml

Boil ten minutes to melt agar, filter through coarse paper. Add 0.7 ml of 0.5 per cent aqueous methylene blue. Adjust pH to 5.8 to 6.0. Tube in 8 cc amounts and autoclave 15 minutes. After cooling, add 1 ml of sterile human serum.

This medium has been used extensively for the cultivation of T. foetus and for this purpose is best adjusted to pH 7.0 rather than 6.0 as is recommended for growing T. vaginalis.

Sprince and Kupferberg (1947a) were the first to publish on a more or less "defined" medium for the sustained growth of T. vaginalis. While this was described as a "simplified medium," the composition as given below indicates this to be in reality a complex medium.

Composition of Complete Trypticase Nutrient Medium for Trichomonas vaginalis

(The amounts listed are the calculated values per 10 ml of final medium)

Trypticase (B.B.L.) . .	200 mg	Riboflavin	8.0 mg
Sodium acetate · $3H_2O$.	48 mg	Thiamine HCl . . .	1.6 mg

Cysteine HCl	15 mg	Pyridoxine HCl . . .	6.4 mg
Maltose	10 mg	Pyridoxamine HCl . .	1.6 mg
Difco agar	10 mg	Pyridoxal HCl	1.6 mg
$NaHCO_3$	5 mg	Ca pantothenate . . .	3.2 mg
Asparagine	2 mg	Nicotinic acid . . .	3.2 mg
Ascorbic acid . . .	1000 mg	p-Aminobenzoic acid .	1.6 mg
Choline chloride . .	80 mg	Biotin	0.8 mg
Inositol	80 mg	Folic acid	0.8 mg
Ribose	40 mg	Methylene blue (optional)	24.0 mg
Adenine SO_4	40 mg	Adjusted to pH 6.0 & Ringer's solution added to make . . .	8.0 ml
Guanine	40 mg		
Xanthine	40 mg	Human blood serum diluted with equal volume of Ringer's solution	2.0 ml
Uracil	40 mg		
		Final volume	10.0 ml

This medium, as pointed out by Sprince and Kupferberg (1947b), was devised primarily for experimental purposes to attempt to assay unknown growth factors in blood serum essential for the sustained growth of T. vaginalis in pure culture. It was not intended as a medium for the routine isolation and propagation of trichomonads. This represented the first report of attempts to quantitate the reproduction of a trichomonad in a more or less chemically defined medium. For the original culture and all serial subcultures, the trichomonad count made after 48 hours incubation at 37 C

averaged 1,344 cells per mm^3 in this medium as compared with 2,047 cells per mm^3 in CPLM medium. The report did not indicate if this represented a significant difference between these two media. There was less variation in cell count from subculture to subculture in the Trypticase medium, the extremes being 915 to 1,655, than in CPLM medium where the extremes were 1,055 to 3,360. It was reported that in the Trypticase medium the cells were more uniform in shape and larger in size and possessed a greater degree of motility.

Kupferberg et al. (1948), while doing additional work on the nutritional requirements of T. vaginalis, developed a comparatively simple medium which has since replaced the more complex Trypticase medium of Sprince and Kupferberg (1947a) for the assay of unknown growth factors. This preparation has been called Simplified Trypticase Serum medium (STS) and has been recommended for isolation and propagation purposes as well as for experimental studies. Its composition as given by Kupferberg et al. (1948) is as follows:

Trypticase (BBL)	20.0 gm
Cysteine HCl	1.5 gm
Maltose	1.0 gm

Agar	1.0 gm
Distilled water	1000.0 ml

The ingredients are mixed and the mixture heated to boiling, cooled, and the pH adjusted to 6.0 for use with T. vaginalis and to 7.0 for use with T. foetus. The medium is tubed in desired amounts and sterilized by autoclaving at 121 C. Prior to seeding, serum in volume necessary to constitute approximately 5 per cent is added to each tube. As indicated by the authors, when the pH was adjusted to 7.0, three strains of T. foetus were successfully carried through serial subculture. Population counts of T. vaginalis through 11 serial subcultures were comparable to those obtained from the original, complex Trypticase medium.

b. Growth in Tissue Culture. Although tissue or cell culture is not used as a method of propagating trichomonads for routine purposes, its use as an investigative method seems perfectly apropos. Hogue (1938) was able to grow three strains of T. foetus, all in pure culture, in whole chicken embryo tissue culture, the suspending medium being 1 part sterile chicken serum to 10 parts of Schumaker's modified Ringer's solution. The tissue culture cells were killed by the trichomonads. Filtrates from 17-day-old

cultures of T. foetus killed the tissue cells as quickly as did living trichomonads. Cultures of T. foetus heated for 15 minutes at 55 C were nearly as toxic as the living flagellates, while cultures heated for 20 minutes at 60-62 C did not affect the growth of the embryonic chicken cells. The tissue culture cells were able to survive in a solution of neutral red which killed the trichomonads.

Hogue (1943) likewise was able to grow T. vaginalis in chicken embryo tissue culture and also in tissue culture from the intestine, lung, and leg muscle of three to five months old human fetuses. The suspending medium (overlay) for the cell cultures would not support the growth of the trichomonads but required, in addition, either liver infusion or tissue culture cells. The tissue cells were destroyed in from 2 to 4 hours after the addition of trichomonads to the culture. When the trichomonad inoculum was very small, tissue cells survived approximately 24 hours. Trichomonads remained actively motile and could be subcultured at least three days after inoculation and always long after the tissue culture cells were dead. As with T. foetus, filtrates of T. vaginalis were also toxic to tissue culture cells, and a drop of filtrate from 5-day-old cultures produced the same changes as when living trichomonads were added.

In neither of the studies reported were trichomonads seen intracellularly.

c. Growth in Embryonating Eggs. Nelson (1938) was the first to report the growth of a trichomonad, T. foetus, in embryonating chicken eggs. He was unable to obtain growth when using the artificial air cell route of inoculation as advocated by Goodpasture for the propagation of certain viruses. Success was achieved when 4-day-old cultures grown in L.E.B. medium were inoculated into 11 to 12-day-old embryos via the allantoic cavity. By subculturing at three to four day intervals, T. foetus was successfully carried through 14 transfers, and there was no evidence that this could not be continued indefinitely. During the period of study, a total mortality of 23 per cent of all embryos was recorded.

Hogue (1939) and Levine et al. (1939) also reported the successful use of embryonating chicken eggs for the growth of T. foetus when inoculation was via the allantoic cavity.

Comparative growth of T. foetus and T. vaginalis in chick embryos was reported by McNutt and Trussell (1941). Following inoculation via the allantoic sac, examination of various embryonic parts demonstrated T. foetus to be present in the allantoic and amnionic fluids but not in the albumin,

yolk sac, the embryo proper, or the blood stream. T. vaginalis did not grow as readily as T. foetus. Six-day-old embryos were most susceptible to infection with T. vaginalis. With T. foetus 12-day-old embryos were optimum. When subcultured into 6-day-old embryos, 24 transfers were made, this being the limit of the experiment.

d. The pH Factor. Media used for the cultivation of trichomonads are adjusted to different initial pH values depending on the species to be grown. As has been pointed out, media for the growth of T. vaginalis are commonly adjusted to pH 6.0 and media for use with T. foetus approximately one pH unit higher. The basis for this is twofold. First, the pH values specified approximate those of the natural environment in which these two respective trichomonads are found. Trussell (1947) indicated that the vaginal discharge in which T. vaginalis is found has an average pH of 5.5. Morgan and Whitehair (1943) reported a varying pH between 6.6 and 8.6 for the uterine pus of cows infected with T. foetus. Second, media adjusted to the values specified have been found experimentally to support maximum cell yield, other conditions being constant.

Cailleau (1934) reported the optimum initial pH for T. columbae in a buffered medium to be 7.0. In such a medium,

the trichomonads lived as long as 34 days at 32 C and, in the same medium but not buffered, a pH of 4.5 existed after five or six days and the acids which accumulated brought about rapid death of the flagellates.

Cailleau (1934) indicated the pH optimum for T. foetus to be in the neighborhood of 7.0 to 7.6 but that reproduction occurred in media where the pH varied from 5.5 to 8.5.

Lyford (1941) found that with T. foetus growing in dextrose-serum broth, there was a concomitant increase in the hydrogen ion concentration and that the extent of the pH change was directly proportional to the number of organisms produced in the culture. She found the final pH of the medium to be approximately the same (pH 5.0) after all the organisms had died regardless of the number produced. She experimented to learn the pH-time values necessary to eliminate motility in a heavy suspension of T. foetus in dextrose-serum broth and found the following relationships:

pH value	Time when motility disappeared
3.5	1 hour and 50 minutes
4.1	2 hours and 20 minutes
4.4	5 hours and 15 minutes
5.0	Still motile after 10 hours

Other workers have reported results which are essentially in agreement with those cited above. Johnson (1940a, 1940b, 1942) indicated T. vaginalis to be very sensitive to the pH of the culture medium. In buffered medium, population increases were inhibited above pH 7.5 and below 5.0. Optimum growth occurred after four days when the pH value was between 5.5 and 5.8. Kupferberg (1940) reported some relation between size of the individual trichomonads and the pH of the culture medium. Under conditions of optimum pH (5.45-5.55), individual cells in 2-day-old cultures were smaller than at any other pH value. He reported the following average sizes at the indicated pH values for 2-day-old cultures:

Length	Width	pH
12.4 $\pm$ 0.13 μ	9.4 $\pm$ 0.11 μ	5.40
15.0 $\pm$ 0.23 μ	11.6 $\pm$ 0.18 μ	7.60

Between these pH values there was a progressive but somewhat irregular increase in size with increase in pH. Morgan (1942) reported a slight variation in the pH of the medium at the peak of cell multiplication for three strains of T. foetus. For the three strains, maximum counts were observed at pH values between 6.1 and 6.3 (Strain A), 5.4

and 5.8 (Strain B) and 5.7 and 5.9 (Strain C). The initial pH for each was 7.2 and the final pH 5.4, 5.2 and 5.3, respectively. Plastridge (1943), reporting a new medium which yielded motile trichomonad (T. foetus) counts as high as 12 X 10^6 per ml, specified that for optimum growth the initial pH should be 7.0 to 7.2.

Kupferberg et al. (1953) followed the pH changes and the cell counts of T. vaginalis in STS medium and noted a pH change from 6.0 to 5.75 in 48 hours with a population increase from 10^4 to 18 X 10^5 cells per ml. Lactic acid was the major acid produced and its production paralleled the observed pH changes. In this study, the human serum that was normally employed for culture purposes was replaced by human serum which had been dialyzed 39 hours against distilled water to reduce the lactic acid content of the serum.

Lindblom (1957) using NIH Thioglycollate Broth at an initial pH of 7.0 to 7.2 demonstrated a peak cell count for T. foetus at approximately 36 hours (4 X 10^6 cells per ml) and a concomitant drop in pH to 6.2, an increase in the titratable acidity and a reduction in the quantity of reducing sugar. After three days incubation, the cell count had dropped to 1.5 X 10^6 per ml, the pH had dropped to 5.5,

the titratable acidity had reached a maximum and the reducing sugar had fallen to approximately one-fourth its original value and one-half that measured at 36 hours.

e. Redox Requirements and Growth in Association with Bacteria. Most trichomonads when encountered in what is commonly considered their natural ecological niche are not observed to be alone but are in association with other protozoa and many bacteria. Yet, by and large, those that are commonly cultured and carried in the laboratory are maintained in axenic culture. M. Lwoff (1951) opined that in their natural environment one of the effects of bacterial metabolism could be to lower the redox potential to an optimum level for the trichomonads.

Not a great deal has been reported on the effect of redox potential on the growth of trichomonads since the early work of Johnson (1940a, 1942). In one of three studies (1940a) it was demonstrated that an environment containing 12 per cent CO_2 exerted an initial reproductive stimulation which was observed after two days incubation. At four days, when cultures were grown in an atmosphere in which hydrogen replaced oxygen completely, there was a significantly greater multiplication than in cultures grown under 12 per cent CO_2 or in air. Johnson (1942)

attempted to quantitate the influence of environmental oxygen tension on the reproduction of T. vaginalis and presented data to indicate that anaerobic conditions were the most favorable for the multiplication of this flagellate.

Read (1953) suggested that an iron porphyrin was involved in the anaerobic respiratory mechanism of T. vaginalis and indicated that under anaerobic conditions this organism metabolized glucose to hydrogen, carbon dioxide and unidentified acids. Ninomiya and Suzuoki (1952) reported that an increase in oxygen tension resulted in a decreased rate of respiration for T. vaginalis. They also demonstrated that this organism showed no catalase activity and postulated that an accumulation of H_2O_2 accounted for the inhibitory effect of oxygen. Read and Rothman (1955) reported that hydrogen gas was produced only under anaerobic conditions of incubation whereas Kupferberg et al. (1953) were unable to demonstrate the production of any gas other than CO_2 in appreciable amounts. These authors reported lactic acid to be the major acid produced.

Comparatively nothing has been done on the effect of redox potential on the multiplication of T. foetus.

Riedmüller (1936) found the growth of this trichomonad in bacteria-free culture to be stimulated by anaerobic cultural conditions but demonstrated by means of the Warburg technique the aerobic utilization of oxygen. Ninomiya and Suzuoki (1952) demonstrated an increased rate of respiration for T. foetus with an increase in oxygen tension. The presence of catalase activity was also demonstrated. Doran (1956) observed a change in the oxygen quotient for T. foetus from a high of 227 µl per 10^8 organisms per hour at 20 hours to 190 at 48 hours to 65 at 120 hours. The acid quotient, measured as CO_2 evolution, likewise was appreciably higher in cultures 20 to 24 hours old than in older cultures and at 120 hours acid formation was not demonstrable. Doran (1957) reported Q_{acid} values for T. foetus nearly three times greater during anaerobic metabolism as compared with aerobic metabolism using glucose as substrate. Ryley (1955) thoroughly studied the metabolism of the Belfast strain of T. foetus and presented the first conclusive evidence for the presence of the Embden-Myerhoff glycolytic pathway.

As stated earlier, most trichomonads in their natural environment are accompanied by a multiple and varied

bacterial flora. It was partly because of this that the axenic cultivation of these flagellates was accomplished only 20 to 30 years ago and that precise metabolic studies have lagged somewhat. Early attempts to cultivate T. vaginalis free of bacteria by Lynch (1915, 1922), Davis (1929), Andrews (1929), and Bland et al. (1932) were unsuccessful.

Morgan (1942) was the first to report the beneficial effect of a single species of bacteria growing in the same medium with T. foetus. He demonstrated an acceleration of growth of this flagellate in the presence of Corynebacterium renale. The exact mechanism was not apparent but the possibility was advanced that there may have been a change in the medium (L.E.B. medium) due to liquefaction and hydrolysis of the proteins and action on the carbohydrate by the bacteria. Plastridge (1943) was unable to maintain T. foetus in culture with staphylococci and coliform organisms. Zeetti (1940) was unable to maintain T. foetus with various species of micrococci. Williams and Plastridge (1946) reported the complete destruction of T. foetus in less than 24 hours by streptococci of the viridans group, Streptococcus fecalis, Streptococcus lactis, Escherichia coli, Aerobacter aerogenes and Proteus vulgaris and in 48 hours

by Staphylococcus aureus and Pseudomonas aeruginosa. Moderate growth occurred with Micrococcus tetragenus, Sarcina lutea, and Bacillus subtilis.

As indicated by Lwoff (1951), bacteria most commonly found in association with T. foetus in the genital tract of the bovine are diplococci, staphylococci, streptococci and probably most frequently Corynebacterium pyogenes. Johansson et al. (1947), by making microscopic counts of T. foetus, found that certain bacteria accelerated, and others inhibited growth. Those that caused a definite inhibition of multiplication were Corynebacterium diphtheriae, C. pyogenes, Corynebacterium xerose, Staphylococcus albus, S. aureus, Streptococcus bovis, S. fecalis, Streptococcus salivarius, a hemolytic streptococcus, Shigella dysenteriae, Shigella paradysenteriae, E. coli and A. aerogenes. Those bacteria which had no pronounced affect or caused a slight acceleration in multiplication were B. subtillis, P. aeruginosa and a diphtheroid isolated from the genital tract of a normal breeding bull. Brucella abortus Strain 19 induced a very rapid multiplication, but these cultures did not survive as long as control cultures. One species, Corynebacterium equi, was outstanding in that motility of trichomonads was prolonged some 288 hours beyond

the 240 hour limit of the pure culture. Bacterial filtrates and heat-killed cultures affected trichomonad growth in a manner comparable to the corresponding viable bacteria. Three possibilities were listed as reasons for the observed differences: (1) changes in hydrogen ion concentration in the culture media, (2) toxin production by the bacteria, and (3) release of additional material favorable or detrimental to trichomonad metabolism as a result of bacterial metabolism.

Pray (1952) reported essential agreement with the results of Johansson et al. (1947). The main point of difference was in the effect of P. aeruginosa, wherein Pray's results were in agreement with those reported by Williams and Plastridge (1946) in that this bacterium caused rapid destruction of the trichomonads. There was no general correlation between the alteration of the pH of the medium and the observed effect on the multiplication of the trichomonads. Likewise there was no correlation between the redox potentials and the effects of the various bacteria.

f. Growth Factors. Since the reporting of pure culture propagation of T. columbae and T. foetus in the early 1930's, some research has been performed on attempting to

identify the components in animal serum without which these flagellates cannot be maintained. When T. vaginalis was obtained in axenic culture by Trussell (1940), another trichomonad became available for this type of research and much of the effort has been with this organism. The first detailed studies on trichomonad growth and nutrition were by Cailleau (1934) who employed three species; viz., T. columbae, T. foetus, and T. colubrorum. As might be expected, it has not been possible to extrapolate completely the results obtained with one species to another species. In fact, it appears there may even be strain differences within a species.

1. Cholesterol, Other Sterols, and Fatty Acids. Cholesterol, or some impurity bound to it, was demonstrated to be essential for the successful propagation of T. columbae by Cailleau (1935). The medium which had been used to cultivate this organism was a peptonized bouillon plus fragments of calf liver. Attempts were made to replace the liver fragments by the liver residue left after extraction with different solvents. Liver extracted by water or ether supported the multiplication of the trichomonads as well as did whole liver. Liver extracted with alcohol supported trichomonad development but at a slower

rate; whereas liver extracted by acetone did not support growth of the flagellates. The acetone extract alone was unable to replace whole liver in the culture medium but when the acetone extract and the acetone residue were both added, growth was maintained. It was concluded that there were two substances in calf liver which were indispensable for the growth of T. columbae: (1) an acetone soluble substance, and (2) an acetone insoluble substance. Attempts to replace the insoluble substance with heat-killed cells of E. coli were unsuccessful. The acetone soluble substance could be replaced with egg yolk, hydrolyzed lecithin, bile, and cholesterol. Ammonium glycerophosphate, oleic, palmitic, and stearic acids could not replace the acetone soluble fraction, and choline hydrochloride and ergosterol were able to support growth for only one subculture. Quantitative studies on the use of an alcoholic solution of cholesterol demonstrated need for a minimum of 1 mg of cholesterol per culture tube in order to obtain abundant cultures (approximately 100 organisms per mm^3). It was pointed out both by the author and by Lwoff (Cailleau, 1935), who commented on this report, that owing to the adsorptive powers of cholesterol there was a

strong possibility that some impurity carried by the cholesterol was the necessary factor rather than the cholesterol itself. Snyder and Meleney(1942) pointed out the probable need for cholesterol in the nutrition of E. histolytica.

Cailleau (1936b) obtained pure cholesterol which was collected from two sources. One specimen of pure cholesterol was obtained from the extracts of the brain of cattle and the other from the extracts of fish liver. A method of purification was followed in which the final step was crystallization three times in the presence of charcoal. This preparation allowed development of the trichomonads in culture when substituted for the acetone extract of liver or for the cholesterol solution reported earlier. The author concluded that cholesterol thus was the first known growth factor to be elucidated for the trichomonads and joined the substance protohemin which had been demonstrated to be indispensable for the cultivation of certain trypanosomes.

In continued studies Cailleau (1936c, 1937b) attempted to determine the degree of specificity of the cholesterol. Cholesterol was replaced by sterols which differed either in the structure of the nucleus or in the constitution of

the side chain. A certain number of modifications of the structure of cholesterol were found to be compatible with the maintenance of activity. Without effect were the following: (1) saturation of the double bond in the 5, 6 position; (2) shifting the double bond from the 5,6 position to the 4,5 position; (3) esterification of the hydroxyl group at position 3; (4) an additional hydroxyl group in the first ring; (5) the presence of a second double bond in the cyclic portion of the molecule; (6) the presence of a double bond in the side chain, and (7) modifications of the side chain which affect only the nature or position of the branches. Those modifications which did result in loss of activity were: (1) modification of the hydroxyl group to a keto group; (2) the presence of a hydroxyl group in position 6 (Ring II); (3) epimerization of the hydroxyl group in position 3; (4) the presence of a ketone group in position 6 or in position 7; (5) removal of the hydroxyl group from position 3; (6) substitution of the methyl group or a Cl atom in position 3, and (7) degradation of the side chain leading to the bile acids or complete elimination of the side chain.

Studies on the cholesterol requirement of T. foetus were conducted by Cailleau (1938a). These studies revealed

that bouillon-calf liver medium sterilized for 20 minutes at 120 C which was adequate for growing T. columbae would not support multiplication of the bovine flagellate. In contrast, when calf liver was replaced by guinea pig liver in the medium, T. foetus grew abundantly and could be subcultured indefinitely. When the guinea pig liver was extracted with acetone or alcohol and the insoluble residue used in the culture medium the results were negative. When cholesterol was added to this, the trichomonads still were unable to reproduce, thus indicating the removal of some essential, not cholesterol or in addition to cholesterol, by the acetone or alcohol extraction of guinea pig liver. Numerous substances were added to the bouillon-acetone extracted guinea pig liver-cholesterol medium such as egg albumin, egg yolk, glucose, bile, yeast extract, Shiga bacilli killed by heating for 15 minutes to 60 C, and living Shigella shiga organisms. Only the medium containing the living bacteria allowed growth of the trichomonad. Using the living Shiga bacilli, the extracted guinea pig liver was no longer needed and T. foetus was able to multiply in serial subculture in a medium consisting of bouillon, living Shiga bacilli, and cholesterol. Removal of the latter substance from the medium resulted in the inability

to maintain T. foetus in this medium. No mention was made of the effect of cholesterol removal on the growth of the Shiga bacillus. The author concluded from these experiments that cholesterol was demonstrated to be a needed growth factor for T. foetus.

Cailleau (1938b, 1939a) demonstrated a need for cholesterol by T. colubrorum and T. batrachorum.

Sprince and Kupferberg (1947b) were the first to report fractionation studies on serum in an attempt to delineate the necessary substances for the growth of trichomonads. They were able to separate serum into an ether-soluble phase and an ether-insoluble phase, both of which were essential for the growth of T. vaginalis. Their studies, in contrast to the findings of Cailleau (1936b, 1938a), indicated that cholesterol was not a growth factor since cholesterol could not replace the ether-soluble extract in culture medium. Estradiol, a female sex hormone previously indicated by Kupferberg and Johnson (1941) to have no affect on the population of bacteria-free cultures of T. vaginalis, was also unable to replace the ether-soluble phase of extracted serum. Substitution of linoleic acid in place of the ether-soluble phase gave, in one instance, an average count of 955 cells per mm^3 on duplicate

determinations on the fifth serial transfer as compared with 1,420 cells per mm^3 in intact serum controls.

More recently, Sanders (1957) has reported the ability of a combination of Tween 80 and cholesterol to replace blood serum in a comparatively well defined medium. He noted that the degree of dispersion of the water-insoluble cholesterol markedly affected growth and that Tween 20, 40, and 60 were inferior to Tween 80 in maintaining growth through several transfers. After six serial transfers, the trichomonad count in the medium containing Tween 80 and cholesterol was 950 per mm^3 while the serum controls showed 1,350 per mm^3.

2. Ascorbic Acid. The growth of various species of trichomonads in nutrient broth enriched with serum from different animal species has been shown to be very irregular. Cailleau (1937c) noted that T. colubrorum would not multiply in a medium consisting of bouillon and rabbit serum but did well when the rabbit serum was replaced by horse serum or guinea pig serum. Trichomonas columbae could not be maintained in a medium of bouillon plus rabbit or horse serum but thrived if the medium contained pigeon serum. Trichomonas foetus grew best in the presence of human serum, guinea pig, or cat serum; whereas, horse and rabbit serum

allowed very poor growth. As pointed out earlier, there was a difference in the growth response of American and European strains of T. foetus. The American (Glaser) strain could be maintained only in a medium with human serum and then only poorly.

There is some evidence that certain animal sera may contain substances which are trichomonicidal. It is possible that this may help to explain the erratic results observed when various animal sera are used with various species of trichomonads. Weld and Kean (1958) tested fresh and inactivated sera from 18 unselected, apparently normal humans, ten guinea pigs, and five rats. In addition sera from hamsters, dogs, and rabbits were tested. Inactivated controls were heated to 56 C for 30 minutes in a water bath. Results were the same with all sera tested. Observed microscopically on a warmed stage, a sequence of events occurred in three steps: (1) loss of motility; (2) flattening and appearance as ghost cells, and (3) complete dissolution. The time to complete lysis varied from three to five minutes. Control specimens remained actively motile for 15 to 20 minutes.

Ascorbic acid was first shown to be contributory to the multiplication of T. colubrorum by Cailleau (1938b)

and to the multiplication of T. foetus also by Cailleau (1938a). Trichomonas foetus would not develop in a medium of bouillon plus cow serum, but the addition of ascorbic acid prepared immediately before use allowed the organism to grow well and to be subcultured. In experiments with T. colubrorum employing a medium consisting of broth, egg albumin, and alcohol-acetone extracted liver, ascorbic acid was found to be confined to the extracted liver.

Cailleau (1939c) likewise demonstrated a necessity for ascorbic acid for the successful cultivation of T. columbae. Fresh pigeon serum added to broth allowed T. columbae to grow abundantly. After ageing 5 months at room temperature it would support the growth of T. columbae only through the first subculture. Addition of ascorbic acid to the aged serum restored its ability to maintain the flagellates in serial subculture.

Ascorbic acid was shown by Johnson (1947) to have a stimulating affect on the reproduction of T. vaginalis. In a medium containing this substance, a cell count of 2,650 per mm^3 was obtained on the third transfer after 72 hour incubation. The same medium without ascorbic acid

yielded only 844 cells per mm.^{3} To date, however, it has not been shown to be indispensable for the growth of this flagellate.

The activity exhibited by ascorbic acid was demonstrated by Cailleau (1939b) not to be specific and could be replaced by D-isoascorbic, D-glucoascorbic, D-glucoheptoascorbic, and reductenic acids and reductone. This lack of specificity renders questionable the role of ascorbic acid and similar substances as growth factors for trichomonads. It is possible that the role of these substances is primarily that of a hydrogen donor or an oxygen acceptor.

Rieck (1953) advocated the use of ascorbic acid in serum-peptone broth medium for the attempted isolation of T. foetus from clinical materials. It was pointed out that population density would not be increased, but maximum growth would be achieved 24 hours earlier. Use of ascorbic acid was especially recommended when the serum used was of equine origin, since serum from this source had been shown to be somewhat variable in its ability to support multiplication of T. foetus. Rieck (1956) reported that in experiments involving attempted isolation of T. foetus from a known carrier bull, using a serum-peptone broth medium, folic acid and pantothenic acid were the only

vitamin B complex members which exhibited a stimulating effect on cell division. A combination of these substances did not increase the rate of division above the level achieved by either of the two used alone, but, upon adding 1 per cent glucose to the combination, a significant increase in multiplication was noted. Ascorbic acid was more reliable than any other water soluble additive tried, but its activity was markedly reduced by the addition of glucose.

3. Pantothenic Acid and Phosphate. A need for pantothenate has been demonstrated only for T. vaginalis. Kupferberg et al. (1948), using a modification of the medium described by Sprince and Kupferberg (1947a), demonstrated that when the amount of serum was reduced from 5 per cent to 0.5 per cent, the trichomonads did not grow on subculture without the addition of calcium pantothenate. Use of serum in 5.0 per cent amounts allowed serial subculture without added calcium pantothenate. When the amount of trypticase was reduced below 0.24 per cent, widely varying populations were observed even with 5.0 per cent serum in the medium. Reduction of trypticase to 0.24 per cent and serum to 0.5 per cent resulted in a complete loss of ability to maintain growth. Inclusion

of 0.027 per cent KH_2PO_4 allowed successful cultivation in low populations through four transfers.

4. Unidentified Factors. A few attempts have been made to replace trypticase in the basal medium of Sprince and Kupferberg (1947a). This pancreatic digest of casein is devoid of carbohydrates, but its exact composition is unknown. Sprince et al. (1949) were able to replace trypticase in a modification of the aforementioned medium by certain pancreas fractions. Merck, Sharp and Dohme's "Al" pancreas fraction and Lilly's "Lipocaic" fraction could replace trypticase in this medium. With the latter substance detectable growth was demonstrated at a concentration of 25 gamma per ml of medium. Known growth factors for other organisms including strepoginin (from insulin), Merck's crystalline vitamin B_{12}, amino acids of fortified casein hydrolysate (Parenamine), and the lipotropic substances, methionine and choline, could not replace the active material from the pancreas. Likewise, crystalline trypsin, and crystalline chymotrypsin were ineffective as replacements.

Sprince et al. (1953) noted that pancreatic extracts were likely to be rich in nucleic acids, or their derivatives, and they conducted experiments to study the effect

of RNA, DNA and their constituents on the multiplication of T. vaginalis. They demonstrated that RNA but not DNA could partially replace trypticase or "Lipocaic" in a modified medium of Sprince and Kupferberg (1947a). The activity of RNA was retained following alkaline or enzymatic hydrolysis but was lost following acid hydrolysis. Sustained growth equivalent to that with RNA was obtained with a combination of either monoribonucleotides (adenylic, guanylic, uridylic, and cytidylic acids) or their respective nitrogenous bases (adenosine, guanosine, uridine, and cytidine). These ribonucleosides, in fact, inhibited growth in media with either free bases or the nucleotides, the order of inhibition being guanosine, adenosine, cytidine, uridine. The inhibition was not evident in media containing trypticase. As pointed out by Hutner and Provasoli (1955), antagonisms among purine bases were known for a number of organisms but that reported by Sprince et al. (loc. cit.), between ribonucleosides and ribonucleotides, for T. vaginalis represented antagonism of a new sort.

g. Carbohydrate Utilization. As for the utilization of sugars, there appears to exist a fairly constant resemblance among the species studied. Cailleau (1934) indicated that T. foetus readily broke down glucose,

galactose, lactose, levulose, maltose, sucrose, raffinose, dextrin, and soluble starch; that inulin was attacked but slightly and there was no utilization of the pentoses, arabinose, xylose, and rhamnose nor the polyalcohols, glycerol, erythrotol, sorbitol, mannitol and dulcitol. Trichomastix colubrorum and T. columbae were observed to have about the same carbohydrate utilization ability with the exception that T. foetus attacked raffinose and strongly acidified levulose and lactose while T. columbae did not attack raffinose and produced only slight acidification from lactose and levulose.

Futumura (1935) came to the same conclusions as Cailleau with regard to carbohydrate utilization by T. foetus and noted, in addition, the acidification of mannose. Riedmüller (1936) confirmed these findings and noted particularly the favorable action of glucose, the fermentation of glycogen and the inability to ferment inositol.

Trussell and Johnson (1941) demonstrated that T. vaginalis utilized only glucose and its polymers, maltose, dextrin, soluble starch, and glycogen to any great extent. Fructose and galactose produced a slight stimulation of growth but no appreciable shift in pH.

Andrews and vonBrand (1938) demonstrated by quantitative studies of the utilization of glucose by T. foetus that a concentration of 0.1 per cent glucose in a serum-broth medium sufficed to meet the requirements of this flagellate. Plastridge (1943) found the presence of a utilizable carbohydrate to be essential in the culture medium and recommended glucose in a concentration of 1 per cent. He was unable to demonstrate the utilization of maltose by the strain he studied. The latter finding is somewhat at variance with the findings of most investigators since maltose is the sugar source in CPLM medium, STS medium, and Sprince and Kupferberg's Trypticase medium. All three media support T. foetus and T. vaginalis in fairly high numbers. Feo (1958) was able to maintain for 20 months and over 80 serial transfers one of two strains of T. vaginalis in STS medium devoid of maltose. These strains had been isolated bacteria-free by using penicillin and streptomycin, from two 74-year-old patients. Counts never exceeded 70 per mm^3 following incubation at 37 C for 72 hours and at times were as low as 7 per mm^3.

h. Use of Serum and Serum Fractions

1. General. As has been mentioned repeatedly, the successful in vitro cultivation of trichomonads in commonly

employed culture media necessitates the inclusion of animal serum. To date there is no exception to this although Sanders (1957) was able to replace serum with cholesterol and Tween 80 in a somewhat complex medium which has not found acceptance for routine use.

The heat stability of the factor, or factors, in serum used for trichomonad cultivation has been referred to by Riedmüller (1936), Cailleau (1937c), Daniel (1940), Schneider (1942), Clausen (1953), and Diamond (1954). Sera heated from temperatures of 56 C to autoclaving temperatures for times varying from 20 to 60 minutes could still support growth of trichomonads. The reports cited have all dealt only with the ability to support growth, there being no attempt at quantitation.

2. Serum Fractions. The use of serum fractions has received very little effort in attempts to isolate and characterize essential substances in serum. This is somewhat surprising in view of the number of serum and plasma fractions commercially available and since the techniques developed essentially by Cohn and coworkers in the 1940's have found universal use and acceptance in commercial and research laboratories throughout the world.

Sprince and Kupferberg (1947a) separated human serum into two fractions by using liquid serum and ethyl ether in a separatory funnel at room temperature. From eight to twelve extractions were made, the number being determined by lack of color extractable by the ether. Ether extraction of frozen serum as reported by MacFarlane (1942) proved unsatisfactory, since the fractions obtained did not emulsify satisfactorily with water, and, as a result, were cumbersome to sterilize by filtration. Neither the aqueous emulsion of the ether extract nor the ether insoluble phase alone supported growth of T. vaginalis. Doubling the volume of the separate fractions added to the basal medium allowed no growth response. Adding the two components simultaneously resulted in a sustained growth through five serial transfers in numbers comparable to those found in five serial transfers using intact serum. (Average = 1,340 cells per mm^3 with intact serum and 1,368 cells per mm^3 with both ether fractions after 48 hours incubation.) Experiments on the ether soluble fraction demonstrated that the active material was heat stable, dialyzable and could be replaced rather effectively by a homogenized aqueous emulsion of a mixture of lecithin, cholesterol, oleic acid, linoleic acid, ergosterol, alpha-estradiol, alpha-

tocopherol, beta-carotene and vitamin A. A plasma lipid fraction prepared by Dr. Jordi Folch-Pi of the Rockefeller Institute Hospital, New York City, also proved to be active. Of the components included in the replacement experiments, linoleic acid appeared to be the most active. In one instance, in which it was used in place of the ether-soluble phase, an average count of duplicate determinations on the fifth serial transfer was 955 cells per mm^3 as compared to 1,420 cells per mm^3 with intact serum controls. The ether insoluble phase could be replaced by 5 per cent human albumin when the ether-soluble phase was used but not when the mixtures of components described above replaced the ether-soluble phase. Sustained growth in subculture was afforded by using albumin and the ether-soluble phase but the cell counts were considerably below those obtained with intact serum. Increasing the concentrations of the ether-soluble extract and the human serum albumin twofold did not effect an increased cell count.

Hardy and Gardiner (1910), MacFarlane (1942), Hewitt (1927), Horsfall and Goodner (1935), and Cappo (1941) all indicated that for the complete extraction of lipids from serum a mixture of alcohol and ether should be used, since ether alone would not suffice. Noting this, Sprince (1948)

altered the separation procedure reported by Sprince and Kupferberg (1947a). Absolute alcohol in a final volume of 13 per cent with respect to serum was added drop by drop to the serum with constant stirring to avoid protein precipitation. This was then extracted with absolute ethyl ether at room temperature by the procedure described earlier (Sprince and Kupferberg (1947a). To facilitate filtration for sterilization Tween 80 was added. In the amount used, Tween 80 had been shown to have no appreciable effect on the growth or inhibition of the strain of T. vaginalis used. The ether-insoluble phase was divided equally into two lots. One portion was sterilized by filtration and represented the complete ether-insoluble phase. The other portion was further fractionated by the drop by drop addition with stirring of absolute alcohol to bring the final alcohol concentration to 50 per cent. This yielded two fractions: (1) a flocculent precipitate and (2) an alcohol soluble portion of the ether-insoluble phase. The former was washed and put into solution by the use of NaOH, the final pH being 7.0 to 7.3. This was sterilized by filtration and contained all the protein precipitable by 50 per cent alcohol. The alcohol filtrate, after evaporation of the alcohol, was adjusted to volume and to pH 7.2 and

sterilized by filtration. This fraction contained all the water-soluble compounds not precipitable by 50 per cent alcohol. None of the fractions alone was able to maintain sustained growth of T. vaginalis. The ether-soluble phase was capable of maintaining survival through three serial transfers from a washed inoculum. This fraction was shown to be free of protein by a number of qualitative tests. When both the aqueous emulsion of the ether extract and the aqueous solution of the solubilized alcohol precipitate of the ether-insoluble phase were added together growth occurred to a degree equivalent to that obtained with intact serum.

The only reference to the use of serum fractions obtained by either the alcohol fractionation method or the ammonium sulfate fraction method was by Sprince (1948). The use of such fractions was referred to only briefly as was the use of intact lyophilized bovine serum. The greatest disadvantage to the use of these materials was the inability to effect complete solubility as evidenced by the loss of material during filtration for sterility.

Clausen (1953) prepared an aqueous extract of inspissated serum by steaming together for 45 minutes equal parts of inspissated serum and distilled water. The extract when

added to 2 per cent glucose broth to give a concentration of approximately 0.15 per cent in terms of its protein content, supported the propagation of T. foetus in serial subculture. The extract could be dialyzed against distilled water without loss of activity. It contained an appreciable amount of protein which could be precipitated by trichloracetic acid or by heating at pH 5.0 but not at pH 7.0. Analysis of the freeze-dried extract and the method of preparation suggested that it might be a seromucoid.

With the aid of pure cultures, slow but steady progress in the study of nutritional requirements and metabolic activities of the trichomonads has been made. As M. Lwoff (1951) pointed out, the pathogenic species awakened the greatest interest and practically all the work reported has been on the three species: T. vaginalis, T. foetus, and T. columbae. von Brand (1950) concluded it was singular in that easily available trichomonads had received so little attention as regards the types of carbohydrate utilized and metabolic pathways followed. Since that time, available reports indicate that some progress has been made and also that much remains to be accomplished.

GENERAL CONSIDERATION OF MATERIALS AND METHODS

I. SPECIES AND STRAINS OF TRICHOMONADS USED.

a. Trichomonas foetus.

1. Strain M. This strain had been carried in artificial culture medium in the laboratories of the Department of Microbiology and Public Health, Michigan State University, East Lansing, since 1954. It was originally isolated from a preputial washing from a bull and was carried until 1958 in either CPLM medium or in Brewer's Modified Thioglycollate Medium (Difco). During the course of the experiments reported herein it was maintained in a slightly modified STS medium. This strain was used throughout the entire study and most of the data were collected using this strain as the test organism.

2. Strain L. This strain was obtained through the generosity of Dr. Norman Levine, College of Veterinary Medicine, University of Illinois, Urbana. It had been isolated from a bull in the Veterinary Clinic at the University of Illinois in December 1957. This strain had been maintained in a frozen condition for some time at the University of Illinois. It was received in CPLM medium

and was transferred to modified STS medium immediately and maintained in this medium for the duration of the experiments.

3. Strain UT. This strain was likewise obtained from Dr. Levine at the University of Illinois. It had been isolated some time earlier by Mr. Paul R. Fitzgerald, Animal Disease and Parasite Research Division, United States Department of Agriculture, Logan, Utah. It also had been kept frozen at the University of Illinois and like Strain L was received in CPLM medium but was transferred to and maintained in modified STS medium.

b. Trichomonas vaginalis. This organism was obtained from Dr. Paul E. Thompson of Parke, Davis and Company, Detroit, Michigan. It was received in CPLM medium and was transferred to modified STS medium adjusted to pH 6.00 and maintained in this medium. Dr. Thompson had obtained this strain from Dr. R. K. Thoms, Department of Pharmacology, University of Connecticut, Storrs, in September 1956. It had been passed intraperitoneally through mice on many occasions by Dr. Thompson.

II. THE BASAL MEDIUM

The most nearly basal medium which has been reported in the literature is the STS medium of Kupferberg et al.

(1948). It was reported to maintain populations of T. vaginalis comparable to those produced in their original assay medium (Sprince and Kupferberg, 1947a). It was also reported to support one strain of T. columbae and three strains of T. foetus. Preliminary trials on the use of this medium revealed an erratic growth response and a more or less steady decrease in the number of motile trichomonads on successive subcultures. In an attempt to stabilize cell multiplication from transfer to transfer, the medium was modified by the inclusion of 0.5 per cent NaCl. The composition of the medium as used for most of the experimental work was as follows:

Trypticase	20.0 gm
Cysteine HCl	1.5 gm
Maltose	1.0 gm
Agar (Bacto)	1.0 gm
Sodium chloride	5.0 gm
Distilled water	1000.0 ml

The distilled water was added to the ingredients in a flask and the mixture brought to a slow boil. It was then cooled in a running water bath to room temperature and the pH adjusted electrometrically using 5N NaOH. For use with T. foetus the pH was adjusted to 7.0 and for use

with T. vaginalis to 6.0. The medium was dispensed into screw cap vials in 20 ml quantities and, with the lids screwed on tightly, was autoclaved at 121 C for 10 minutes. It was allowed to cool to room temperature and was then stored in the refrigerator at approximately 4 C. Upon standing crystallization was observed to occur at a level approximately 0.5 to 1.0 cm subsurface. This became apparent after about two weeks of storage and necessitated the frequent making of new batches of the medium. Subsequent experimentation during which cysteine was omitted from the medium demonstrated no such crystal formation in this medium.

This medium was used throughout the study both for routine maintenance of the trichomonad strains and for assay purposes.

III. ENUMERATION OF TRICHOMONADS.

Cell counts were used throughout for evaluating the ability of a given fraction to replace intact serum in the completed culture medium. Appropriate dilutions were made in 0.8 per cent NaCl and the trichomonads counted in a Spencer Bright Line haemocytometer. The large corner squares, used for enumerating white blood cells when doing blood counts, were used and a 10 X phase contrast (dark M-AO)

objective in combination with 10 X wide field oculars was employed. With this arrangement all 16 smaller squares in each of the larger corner squares could be viewed at one time. Only trichomonads that demonstrated some degree of motility were counted. Motility exhibited by young and vigorous organisms caused no difficulty in making counts. At the magnification used and with the normal degree of motility exhibited by trichomonads they were easily enumerated.

All assays were made in duplicate and duplicate counts were made on each tube. Preliminary testing revealed essential agreement between counts obtained by this procedure and counts obtained when six to eight counts were made per tube. Each of the counts recorded represents the mean of four counts, two from each of the duplicate tubes.

Statistical analysis of the data was performed by analysis of variance (F test) following essentially the procedures of Goulden (1952).

IV. INCUBATION TIME AND ENVIRONMENT

In most culture media peak populations of trichomonads are observed from 36 to 92 hours after inoculation. As a rule this can be narrowed to 48 to 72 hours but there is some variation. Usually by five days the population of

motile organisms has declined appreciably, especially if 37 C incubation temperature has been maintained.

A majority of counts were made in these experiments after 72 hours of incubation. This was done for two reasons: (1) Counting at 24 hour intervals revealed peak populations usually at 72 hours, at least when intact serum was used. When certain serum fractions were used there was a noticeable increase in trichomonad count between 48 and 72 hours of incubation. (2) From the standpoint of time involved it was convenient to make only one count during the incubation period. With these coming at 72 hour invervals it was possible to set up three series during a given assay period and only one of the three series would be considered on any one day. For some experiments counts were made after 48 hours and 120 hours incubation.

Incubation was carried out in all experiments at 36 C to 37 C, this being the optimal temperature for both T. foetus (Cailleau, 1934; Witte, 1933) and T. vaginalis (Johnson and Trussell, 1944).

As pointed out earlier T. vaginalis has as one of its culture requirements some degree of anaerobiasis. This is the reason for the inclusion of small amounts of agar in most media and also the reason for the inclusion of the common reducing agent cysteine. Trichomonas foetus, on

the other hand, appears to be facultative in this regard although most researchers have felt that better growth is obtained if some degree of anaerobiasis is maintained. For the latter reason, and because of results obtained in early preliminary experiments, a reduced oxygen tension was employed for all incubations. This was accomplished by placing the screw cap culture tubes, with caps ajar, in a glass desiccator and by means of a vacuum pump removing the air until a pressure of approximately 50 mm of Hg was obtained in the jar. This pressure was chosen only because at a lower pressure degassing of the medium was apt to be so violent as to cause the medium to boil out of the culture tubes. This will be referred to as anaerobic incubation as opposed to aerobic incubation where cultures were held under existing atmospheric conditions.

V. BLOOD FRACTIONS USED.

a. Serum.

1. Human Serum. During the early part of the work when human plasma fractions were being used intact human serum was used as the control. This was obtained through the courtesy of Dr. Keith McCall, Bureau of Laboratories, Michigan Department of Health, Lansing. It was obtained as citrated plasma which was harvested from out-dated blood

at the Department's Blood Bank. A few crystals of calcium chloride were added to the plasma to cause it to clot. Following clotting the serum was harvested and sterilized by filtration through Hercules ST-3, Seitz-type filter pads.

2. Horse Serum. Horse serum was used as the control in most of the experiments. After the initial experiments using commercially available human plasma fractions, horse serum was used throughout, both for controls and for fractionation procedures conducted in the laboratory. This serum was obtained as fresh serum through the generosity of Dr. L. P. Hedeman of the Bureau of Laboratories, Michigan Department of Health, Lansing. It was received in approximately gallon lots and had been harvested from blood taken from normal, uninoculated animals in the Department's Biological Production Section. Immediately after receipt the serum was sterilized by passage through Hercules ST-3, Seitz-type filter pads.

All serum was inactivated by heating to 56 C for 30 minutes in a water bath immediately after sterilization. It was stored in the refrigerator at approximately 4 C until used.

b. Commercially Prepared Plasma and Serum Fractions.

1. PPLO Serum Fraction. Sterile PPLO Serum Fraction was purchased from Difco Laboratories, Detroit,

Michigan. Two lots were employed, control number 431914 and control number 438867. This fraction was developed by Smith and Morton (1951) for use in the cultivation of certain pleuropneumonia-like organisms. This substance had also been shown by Rose and Morton (1952) to support the *in vitro* cultivation of three strains (Nichols, Reiter, and Kagan) of avirulent *Treponema pallidum*. As described by Smith and Morton (*loc. cit.*) two materials were obtained from their procedure both of which would support growth of the PPLO organisms. Fraction A was obtained from human ascitic fluid and horse and beef serum when fractionated with ammonium sulfate at a concentration between 0.62 and 1.00 saturation according to the method of Cohn *et al.* (1940a). Heating Fraction A in a boiling water bath at pH 7.0 for 20 minutes and then adjusting the pH to about 4.0 with hydrochloric acid and removing the coagulum showed all the activity to be in the filtrate which was designated Fraction B. It was concluded from their studies that the growth factor for the PPLO organisms in ascitic fluid and serum was not carbohydrate or lipid but protein in nature. Its properties suggested that it might be a protein of low molecular weight or a large polypeptide derivative.

Correspondence with Mr. S. H. Davis and Mr. A. L. Lane of the Difco Laboratories failed to reveal whether their commercial product corresponded to Smith and Morton's Fraction A or Fraction B. Since it was observed to coagulate on heating to 100 C in a boiling water bath, which according to Smith and Morton (loc. cit.) Fraction B will not do, it was assumed to be Fraction A.

2. Human Plasma Fractions. Human plasma fractions prepared by the alcohol fractionation method of Cohn et al. (1940b) were generously supplied by Dr. Keith McCall, who also supplied the human plasma.

Fractions I, II + III, IV-1, IV-4, and V were received as wet pastes as they came from the fractionation procedure. These pastes were distributed into separate wide mouth glass bottles, quick frozen in an alcohol-dry ice bath at -73 C and lyophilized at a manifold pressure of approximately 100 μ Hg for 48 hours. Following drying, screw caps were placed tightly on the jars and the fractions were stored at 4 C. Fraction VI, the final liquid remaining after the last precipitate was removed, was lyophilized at the Laboratories of the Michigan Department of Health. This was done since the volume of liquid to be processed was of such a magnitude that commercial drying equipment was needed.

Cohn (1945) has indicated the composition of the various plasma fractions obtained by the alcohol-fractionation method. Fraction I consists essentially of fibrinogen and fibrinolytic enzyme; Fraction II, gamma globulins; Fraction III, gamma globulins including euglobulins, the isoagglutinins, beta-globulins, cholesterol, carotenoids, phosphatases and phosphatides; Fraction IV-1, alpha-globulins, cholesterol and phosphatides; Fraction IV-4, beta-globulins, alpha-globulins and hypertensogin; Fraction V, albumin; and Fraction VI, alpha-globulin and gonadotropic hormones.

Sprince (1948) alluded to the difficulty in sterilizing by filtration serum fractions prepared by the Plasma Fractionation Laboratory at the Harvard Medical School. This resulted actually from a lack of complete solubility of the various fractions. A similar difficulty was encountered in the work reported herein and ultimately work with these fractions was discontinued due to this difficulty. The use of 0.8 per cent NaCl solution for attempted solubilization of the various fractions was of some help. The dried fractions were prepared for use in a concentration reported by Cohn (1945) to be the protein concentration for that particular fraction in whole plasma. Fraction I

was reported to account for 4.3 gm of a total of 60.3 gms of protein per liter of plasma, Fraction II + III, 16.3 gm, Fraction IV, 9.7 gm, Fraction V, 29.6 gm and Fraction VI, 0.6 gm. Accordingly the fractions were prepared for use as follows:

Fraction	Amount used in gm/100 ml 0.80 per cent NaCl
I	0.5
II + III	1.75
IV-1	1.0
IV-4	1.0
V	3.0
VI	0.075

Each fraction was prepared in a concentration slightly higher than it had been reported in whole plasma. This was done in an attempt to get into solution at least the amount normally found in plasma. Fractions I, V, and VI were readily soluble in saline or in distilled water but Fractions II + III, IV-1, and IV-4 were practically insoluble in distilled water and went into solution in saline only with great difficulty. Each preparation was mixed with a magnetic mixer at room temperature for a maximum of 30 minutes. Following this they were centrifuged in

an International Equipment Company Clinical Centrifuge for 15 minutes at 1500 rpm. The supernate was passed through a Hercules C-5, Seitz-type filter pad for clarification and finally through a Hercules ST-3, Seitz-type pad for sterilization. All fractions were stored at 4 C.

c. Laboratory Prepared Fractions.

1. Heated Serum. Two different procedures were followed in the experiments with heated horse serum. In one procedure, sterile horse serum was heated to approximately 100 C for 20 minutes in a water bath after which time the liquid portion was removed by means of a pipette equipped with a rubber bulb. The liquid was removed aseptically from the tube before removing the latter from the bath in order to prevent the liquid from being taken into the coagulum, which would occur on the slightest cooling. The volume of this liquid was restored to the volume of the original serum by the addition of sterile 0.8 per cent NaCl. This was stored at 4 C until used.

A second experiment employed whole serum which had been added to the basal medium in 5 per cent concentration (0.25 ml serum in a total volume of 5.00 ml) and the tubes ready to be inoculated were autoclaved at 121 C for 10 minutes. By carefully depositing the serum on the bottom of the

tube and taking care not to mix it with the medium, the coagulum formed on autoclaving was in the form of a thin shell conforming to the contour of the tube. Thus the center of the medium was completely liquid, and this greatly facilitated obtaining samples for counting and transferring. Tubes were placed in a running cold water bath as soon as they were removed from the autoclave and when cool were inoculated immediately.

2. Dialyzed Horse Serum and Horse Serum Dialysate.

A. Dialyzed Horse Serum. Two procedures were followed in the experiments with dialyzed horse serum. All dialyses were begun with sterile horse serum and were carried out at 4 C to guard against bacterial growth. All dialyses were against distilled water and were done using cellophane tubing to contain the serum, with continuous stirring supplied by an American Instrument Company Synchronous Agitator.

One batch of serum was dialyzed for a total of 18 days. The distilled water against which the serum was dialyzed was changed two, three, or four times a day during the 18 day period. Marked precipitate formation was observed in the serum sample on day 12. After the 18 day dialysis period the precipitate was washed by centrifugation in

three changes of distilled water. It was put into solution by the addition of 0.8 per cent NaCl solution and brought to the volume of the original serum sample. The liquid portion of the dialyzed serum was placed in a cellophane bag and hung overnight in the refrigerator in front of a fan. This reduced the volume to slightly less than the original serum volume so that distilled water had to be added to return it to the desired volume. Sodium chloride in a final concentration of 0.8 per cent was added to this. The two fractions thus obtained were designated precipitate fraction and water soluble fraction. They were sterilized by filtration and stored at 4 C until used. They were assayed individually and together.

A second serum dialysis was conducted which was allowed to proceed for only 72 hours. Two hundred ml of horse serum were dialyzed against 600 ml of distilled water. During the 72 hour period of dialysis the distilled water was changed a total of eight times. The dialysate, a volume of 5400 ml, was lyophilized for 48 hours using commercial drying apparatus at the Michigan Department of Health. The serum contained a slight precipitate which was readily solubilized on the addition of NaCl crystals. This, designated as 72 hour dialyzed horse serum, was

sterilized by filtration and was stored at 4 C.

B. Horse Serum Dialysate.

Lyophilization of the horse serum dialysate yielded a light, white, flaky powder which was readily soluble in water and 0.8 per cent NaCl. The powder was dissolved in 200 ml of distilled water and then 1.6 gm of NaCl were added. The solution was sterilized by filtration and stored at 4 C.

3. Lipid Solvent Extractions.

Sprince and Kupferberg (1947b) extracted liquid serum at room temperature with ethyl ether containing 0.1 per cent or less of alcohol and obtained ether-soluble and ether-insoluble fractions both of which were essential for maintaining T. vaginalis in culture. Sprince (1948) reported an improvement in this procedure which involved pre-treatment with absolute alcohol in a manner to avoid protein precipitation. This was followed by extraction with ethyl ether of the same purity as used in the original work. Smith and Morton (1951) in their studies on growth factors required by the pleuropneumonia-like organisms extracted lyophilized serum at room temperature with ethyl ether, benzene and chloroform.

Two extraction procedures were followed in the present

study. Horse serum was lyophilized in 50 ml amounts for 48 hours at a manifold pressure of approximately 100 μ Hg. Extraction of 50 ml of serum, after drying, was done at room temperature using diethyl ether (Merck and Company) containing 3.5 per cent alcohol. A total of ten extractions was made, using 100 ml of ether per extraction. Each extraction was periodically shaken and was allowed to proceed for 30 minutes. The ether soluble material was discarded. The ether was removed from the residue by evaporation *in vacuo*. Following this the residue was reconstituted to its original volume by the addition of distilled water and the reconstituted ether extracted serum sterilized by filtration.

The second extraction procedure was performed quite differently. MacFarlane (1942) indicated that freezing serum to -23 C resulted in a dissociation of the lipid-protein complexes and a liberation of the fatty materials. He suggested the extraction of serum after freeze drying since the reconstitution of such extracted serum with water formed a clear solution and there was no evidence that the physical properties of the serum protein were affected by this procedure. Accordingly the dried serum residue from 50 ml volumes of serum was used. Four such

samples were placed in asbestos mantels for use in Soxhlet reflux extractors and were extracted for 24 hours with diethyl ether (Merck and Company) containing 3.5 per cent alcohol, acetone, benzene or chloroform (Mallinckrodt, Analytical Reagent). Solvents were used in 300 ml volume and extractions were performed in a chemical hood using a sand bath and hot plate as the heat source. The heat was adjusted to allow gentle boiling of the solvent.

Following the extractions the solvents were removed by evaporation in vacuo. The insoluble residues were reconstituted to original volume by the addition of distilled water and were sterilized by filtration. The solubles were taken up in 50 ml of 5 per cent serum albumin (Human Fraction V of plasma fractions previously described) and sterilized by filtration. All fractions were held at 4 C.

4. Ammonium Sulfate Fractions.

Following the difficulties encountered in the reconstitution of certain commercial fractions of human plasma prepared by the ethanol fractionation procedure, fractionation by the "salting-out" technique was tried. Both ammonium sulfate and sodium sulfate have been used widely for fractionation by this procedure. Sodium sulfate has the disadvantage of having to be used at 37 C because its

solubility at room temperature is not sufficiently high. Ammonium sulfate has a very high solubility and performs well at room temperature, but contains nitrogen so that direct Kjeldahl analyses of the protein fractions are rendered impossible (Haurowitz, 1950). Since nitrogen determinations were not contemplated and since it was convenient to work at room temperature, fractionation using ammonium sulfate was followed.

The procedure followed for ammonium sulfate fractionation was essentially that of McMeekin (1939, 1940), and Cohn et al. (1940a).

The apparatus as used is schematically represented in Figure 1. This consisted of a Pyrex glass jar into which was placed the diluted serum sample. Cohn et al. (loc. cit.) indicated that fractionation by this method was slightly more effective if the proteins were in a somewhat lower concentration than they are in serum. Originally 350 ml of serum and 100 ml of distilled water were placed in this container. This solution was stirred by a teflon covered magnet which was propelled by a stirring motor positioned immediately beneath the glass jar. Since this motor in operation generated an appreciable amount of heat it was necessary to insulate the glass

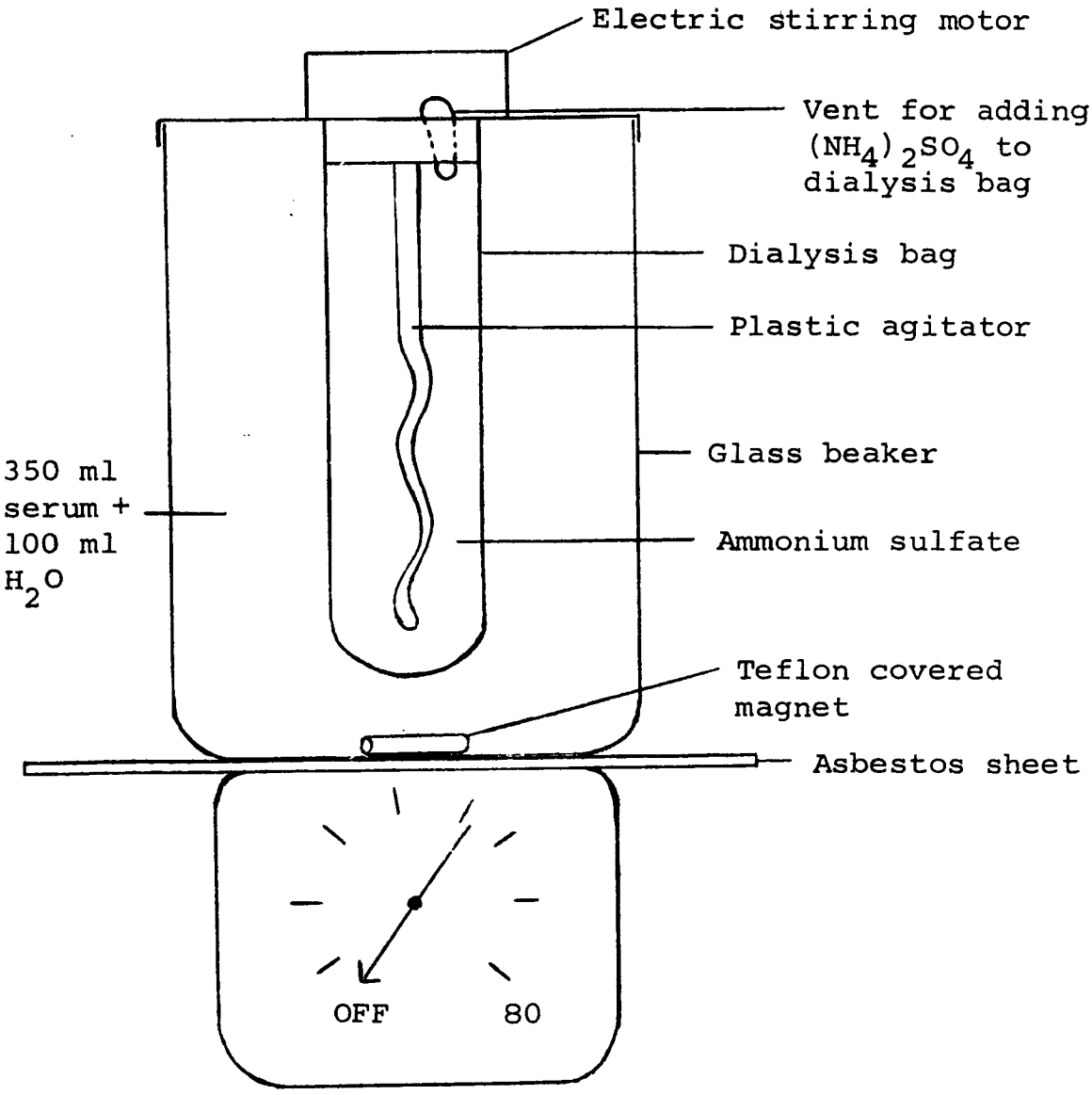


Figure 1. Schematic diagram of apparatus used for the fractionation of horse serum by the ammonium sulfate method.

container from it. This was very effectively accomplished by the use of 1/8 inch thick sheet asbestos. Ammonium sulfate (Mallinckrodt, Analytical Reagent) was placed in the cellophane bag which was suspended in the serum solution by a plate covering the top of the glass jar. Distilled water was added to this bag, as were ammonium sulfate crystals at subsequent times, through an opening in the top of the metal plate thus avoiding the necessity of removing the cellophane bag for this purpose. The volume of liquid in the apparatus was measured at each addition of ammonium sulfate in order to know the exact amount of salt to add. After each addition of salt 24 to 36 hours were allowed for precipitation of the fraction insoluble at the particular salt concentration being used. At this time the serum solution in the outer jar was filtered through Whatman Number 42 filter paper supported in a Buchner funnel and the filtrate returned to the dialysis jar. The precipitate was thrice washed using 50 ml volumes of the particular ammonium sulfate concentration at which it had been precipitated. A total of six fractions were collected. Five of these were precipitates and the sixth was a liquid representing the substances soluble in 2.8 molar ammonium sulfate. The six fractions and the amount

of salt used in obtaining these fractions are indicated in Table 1.

Table 1. Serum fractions prepared by the salting-out method employing ammonium sulfate

Fraction designation	Concentration $(NH_4)_2SO_4$ in moles/liter	Per cent saturation
1	1.39	0.34
2	1.64	0.40
3	2.05	0.50
4	2.57	0.62
5	2.80	0.68
6	Solubles in 2.8 M $(NH_4)_2SO_4$ (0.68% saturation)	

Fractions 1 through 5 were handled individually as follows. They were placed, along with the filter paper on which they were collected, in 600 ml beakers and distilled water was added. The preparation was agitated during the addition and until all traces of the precipitate disappeared. The final volume of each of the fractions was brought to 400 ml. Fraction 6, which was collected as a liquid, was from this point on handled exactly as were the first five fractions which were collected as precipitates. All fractions

were transferred to cellophane dialyzing tubing and dialyzed at room temperature for approximately 96 hours against cold running tap water. At this point the material in the dialysis bag was tested and found negative for sulfate ions by the barium chloride method. To five ml of each fraction was added four drops of concentrated hydrochloric acid and 2.5 ml of 0.5 M $BaCl_2$. The absence of a white precipitate ($BaSO_4$) formation indicated removal of the ammonium sulfate by the dialyzing process. Each fraction was further dialyzed for 24 hours against distilled water in the cold with four changes of the dialysate during this period. Following this each fraction in its cellophane dialysis bag was hung in front of a fan at 4 C for 36 to 48 hours. The resulting evaporation reduced the volume to approximately 200 ml. The fractions were then transferred to graduate cylinders, 2.8 gm of NaCl added to each and the volume adjusted to 350 ml with distilled water. All fractions thus obtained were perfectly clear, fractions 1 through 5 having the appearance of water, and fraction 6 having the typical color of the original serum. Approximately 100 ml of each fraction were sterilized by filtration through Hercules ST-3, Seitz-type filter pads and stored at 4 C. The remaining portions of the fractions were stored frozen at approximately -20 C.

5. Alcohol Precipitation.

Complete protein precipitation is commonly accomplished by the addition of 95 per cent ethanol in a ratio of 3:1 alcohol to serum. One hundred fifty ml of 95 per cent ethyl alcohol were added drop by drop from a burette to 50 ml of horse serum. The serum was agitated continuously by means of an electric stirrer. The resulting precipitate was removed by filtration through number 42 Whatman filter paper and was discarded. The liquid was placed in cellophane tubing and suspended in front of a fan at 4 C for approximately 72 hours to remove the alcohol. The final volume was adjusted to 50 ml and sterilization was accomplished by filtration. Storage was at 4 C.

d. Hemoglobin.

Hemoglobin freshly prepared from equine erythrocytes and commercially prepared hemoglobin (Difco) were reported by Smith and Morton (1951) to contain the growth promoting factor for pleuropneumonia-like organisms. Riedmüller (1936) reported that with a broth base, red cells washed or hemolyzed, were devoid of activity for supporting growth of T. foetus.

Two per cent hemoglobin was prepared from Baker's Hemoglobin Powder (purified, origin species not specified),

in 0.8 per cent NaCl. It was sterilized by filtration and stored at 4 C.

VI. SUPPLEMENTARY ADDITIVES.

a. Pantothenic Acid. Pantothenic acid was used in the form of its calcium salt obtained from Nutritional Biochemical Corporation. Kupferberg et al. (1948) had shown a requirement for pantothenic acid by T. vaginalis when the serum concentration in STS Medium was reduced from 5 per cent to 0.5 per cent. Denko et al. (1947) had reported that normal whole blood has on the average 33 µg of pantothenic acid per 100 ml. The solution for use was prepared by adding 640 mg of calcium pantothenate to 100 ml of distilled water. This was thoroughly shaken and 0.5 ml of this solution (= 3.2 mg) diluted to 100 ml, thus giving a solution which contained 32 µg per ml. This constituted the solution for use and was sterilized by filtration and was stored at 4 C. Since it is stable on storage under these conditions it was necessary to make this preparation only one time. It was used in the culture medium in certain experiments in volume necessary to supply the vitamin at a level of 3.2 µg per 10 ml of medium.

b. Ascorbic Acid. Rieck (1953) indicated that ascorbic acid reaches its highest effect at concentrations between 0.15 and 3.5 mg per ml of culture medium. Ascorbic acid powder (Wyeth, Incorporated) was used in experiments reported herein. It was made up in 10 per cent concentration in distilled water and was sterilized by filtration. It was stored in the dark at 4 C. Since vitamin C is unstable and deteriorates fairly rapidly even under the conditions specified it was prepared fresh at weekly intervals. It was used at a level of 0.05 ml per 5.00 ml total volume in the culture tubes, thus giving a concentration of 1.0 mg per ml.

c. Cholesterol and Tween 80. Cholesterol solution was prepared by the Hartman-Leddon Company, Philadelphia, and was obtained from the Fisher Scientific Company. It was an anhydrous chloroform solution and contained 1 mg of cholesterol per ml. It was stored in the dark at 4 C and for dilution purposes was diluted with anhydrous chloroform (Mallinckrodt, Analytical Reagent).

Tween 80 (polyoxyethylene sorbitan monooleate) was obtained from the Chemicals Department of the Atlas Powder Company, Wilmington, Delaware. Dilutions were prepared fresh as used in 70 per cent ethanol (v/v) as specified by Sanders (1957).

Cholesterol and Tween 80 were added to the basal medium, without serum, and the tubes with screw caps ajar were autoclaved 10 minutes at 121 C. After exhausting the autoclave slowly the hot tubes were allowed to cool at room temperature by setting on the laboratory bench. All traces of chloroform, as evidenced by lack of chloroform odor coming from the tubes, were removed by this procedure.

VII. ASSAY PROCEDURE.

Assays of all substances tested were performed in the Simplified Trypticase Medium of Kupferberg et al. (1948) modified by the addition of 0.5 per cent NaCl (see p. 54). The original seeding of a given series was from a 48 or 72 hour culture of the test organism in the same medium containing serum in 5 per cent concentration. All seedings, original culture and subsequent subcultures were in volume necessary to transfer approximately 50,000 trichomonads to a final volume of 5 ml per tube so that the concentration of organisms was approximately 10 per mm^3. This was felt necessary in that it is known that the number of organisms at a given time post inoculation is in some measure related to the number of organisms in the inoculum. Counts were made at 48, 72 and/or 120 hours post inoculation as indicated.

For a given test material to be considered contributing to the support of the trichomonads in serial subculture, an average count of 50 motile organisms per mm^3 was arbitrarily chosen. In order to satisfy the requirement of 50,000 organisms needed for transfer, counts of 50 cells per mm^3 necessitated transferring 1.0 ml. Cultures containing substances showing evidence of supporting sustained growth under these circumstances were counted for the original inoculation and for five successive serial transfers. Serum substitutes were tried in varying concentrations since serum in varying concentration has a marked affect on the number of trichomonads in a culture medium at a given time post inoculation. All fractions were tested by inoculating duplicate tubes at each transfer. Duplicate counts were made of each tube and the final average was computed from the average counts of each transfer. The same basal medium containing 5 per cent intact serum served as the control and in final analysis all fractions were compared with it.

VIII. FRACTION IDENTIFICATION.

A moderate attempt was made to qualitatively characterize the chemical nature of certain of the fractions which gave evidence of supporting sustained growth. This was an

attempt to determine if these fractions contained carbohydrates, fats and proteins.

a. Test for Carbohydrates. The Molisch test was used to determine the presence or absence of carbohydrate in a given sample.

b. Tests for Sterols. The Liebermann or Liebermann-Burchard Test and the Salkowski Test were performed to check for the presence of sterols. According to Gortner (1949) these tests are apparently specific for the unsaturated sterols.

c. Tests for Proteins. The qualitative tests used to detect the presence of proteins were: (1) Coagulation by heating to 100 C; (2) Biuret Test; (3) Heller's Test (concentrated nitric acid), and (4) Trichloracetic Acid.

d. Analysis by Ultracentrifugation and by Electrophoresis. Qualitative ultracentrifugation analysis was conducted on two of the fractions and paper electrophoresis analysis on one of the fractions. These analyses were conducted to obtain some idea of the homogeneity of these fractions.

1. Ultracentrifugation.

The PPLO serum fraction obtained from Difco and fraction 5 obtained by ammonium sulfate fractionation were

analyzed in a Spinco Model L ultracentrifuge. The data for each of the fractions are as follows: (1) PPLO fraction; speed 59,780 rpm, voltage 80, photographs taken with 6 seconds exposure at 16 minute intervals. (2) Ammonium sulfate fraction 5; speed 58,880 rpm, voltage 80, photographs taken with 3.5 seconds exposure at 8 minute intervals.

2. Electrophoresis.

Electrophoresis using "oxoid" (Consolidated Labs., Inc., Chicago, Illinois) cellulose acetate strips according to the method described by Kohn (1957a, 1957b) was carried out in the cold for approximately 3 hours.

EXPERIMENTAL RESULTS

I. THE EFFECT OF THE ADDITION OF 0.5 PER CENT NaCl TO THE BASAL MEDIUM WITH SERUM ADDED.

Preliminary work attempting to learn if incubation should be under atmospheric conditions or at reduced oxygen tension demonstrated an erratic growth pattern and a somewhat steady decline in the number of motile trichomonads on successive subculture. Table 2 gives the results of these trials. It is clearly evident that there was a wide variation in the number of trichomonads from subculture to subculture whether incubation was aerobic or anaerobic. The mean cell count per mm^3 per culture, however, was nearly twice as great under conditions of reduced oxygen tension as under aerobic conditions. Subsequent experiments using PPLO serum fraction in place of whole serum confirmed this observation and as a result all subsequent incubation was done anaerobically.

Most culture media which do not use a salt solution such as Locke's solution or Ringer's solution usually have NaCl added in concentrations varying from 0.4 to 0.8 per cent. The basal medium of Kupferberg *et al.* (1948) calls for no NaCl and it was felt this might be one reason for the variable and low counts observed in the preliminary experiments on incubation environment. Accordingly tests were

conducted to learn if the addition of NaCl in 0.5 per cent concentration would have any affect on the number of trichomonads per culture on serial subculture. Table 3 shows the results of these experiments. The influence of salt in the culture medium is readily apparent since the medium containing salt, while showing variation from subculture to subculture, showed a mean cell count of 1253 per mm^3 at 48 hours as compared with 485 in the medium without salt, and, 1988 cells per mm^3 at 120 hours as compared with 665 in the medium without salt. It is obvious that these are statistically highly significant differences.

Table 2. Comparative growth of T. foetus strain M in Basal Medium without NaCl and with 5 per cent serum added. Incubated under atmospheric conditions (aerobic) and at a pressure of approximately 50 mm Hg (anaerobic).

72 Hour Cell Count per mm^3

	Original culture	Subculture number									Mean
		1	2	3	4	5	6	7	8	9	
Aerobic 5% horse serum	725	875	400	875	813	400	425	169	650	225	556
Anaerobic 5% horse serum	975	1525	1050	1075	2400	750	138	1050	413		1042

To learn if this was a constant pattern, reciprocal medium transfers were made from the fifth subculture of the original experiment. Cells which had been growing in the medium without salt were transferred to the medium with salt and cells growing in the medium containing salt were transferred to the medium without salt. The results of this trial are shown in Table 4.

Table 3. Comparative growth of T. foetus strain M in Basal Medium with and without added NaCl.

48 Hour Cell Count per mm^3

	Original culture	Subculture number									Mean
		1	2	3	4	5	6	7	8	9	
Without salt	325	650	1000	300	375	750	475	325	400	250	485
With salt	1750	1350	950	1225	950	1300	1800	1300	875	1024	1253

120 Hour Cell Count per mm^3

	Original culture	Subculture number									Mean
		1	2	3	4	5	6	7	8	9	
Without salt	500	1225	1075	475	250	1225	725	450	450	275	665
With salt	1650	1450	2600	2625	2450	2025	1350	1675	1600	2450	1988

Table 4. Comparative growth of T. foetus strain M in Basal Medium with and without added NaCl. Inoculum for medium with NaCl from 5th subculture in medium without NaCl and, inoculum for medium without NaCl from 5th subculture in medium with NaCl.

48 Hour Cell Count per mm^3

	Original culture	Subculture number					Mean
		1	2	3	4	5	
Without salt	750	325	175	175	450	375	375
With salt	1250	900	1400	700	650	1300	1033

120 Hour Cell Count per mm^3

	Original culture	Subculture number					Mean
		1	2	3	4	5	
Without salt	1175	400	350	450	325	525	538
With salt	2200	3125	3050	1350	2025	2100	2308

Here again the results are clearly indicative of the beneficial effect of 0.5 per cent NaCl added to this medium since the mean number of trichomonads per mm^3 of culture both at 48 and at 120 hours incubation is significantly greater in the medium with added salt.

For use in the remainder of the experiments the basal Simplified Trypticase Medium of Kupferberg et al. (1948) was modified to contain 0.5 per cent NaCl.

II. PPLO SERUM FRACTION (DIFCO) AS A REPLACEMENT FOR SERUM.

PPLO Serum Fraction (Difco) is a commercially available preparation which is that part of serum precipitated between 0.62 and 1.00 ammonium sulfate saturation. Since it has been recommended for the in vitro cultivation of the fastidious pleuropneumonia-like organisms and also for the avirulent strains of T. pallidum it was felt it might be capable of replacing intact serum for the maintenance of T. foetus. Accordingly experiments were run to learn if this might be accomplished. Results of these experiments are shown in Figure 2.

Varying the concentration of horse serum and PPLO serum fraction in the final medium from 1 to 2 to 5 to 10 to 20 per cent and making counts after 48 and 120 hours incubation demonstrated the PPLO serum fraction capable of supporting growth of T. foetus in serial subculture in all concentrations tested. Forty-eight hour counts from tubes containing any of the concentrations of horse serum were appreciably higher than those in the corresponding

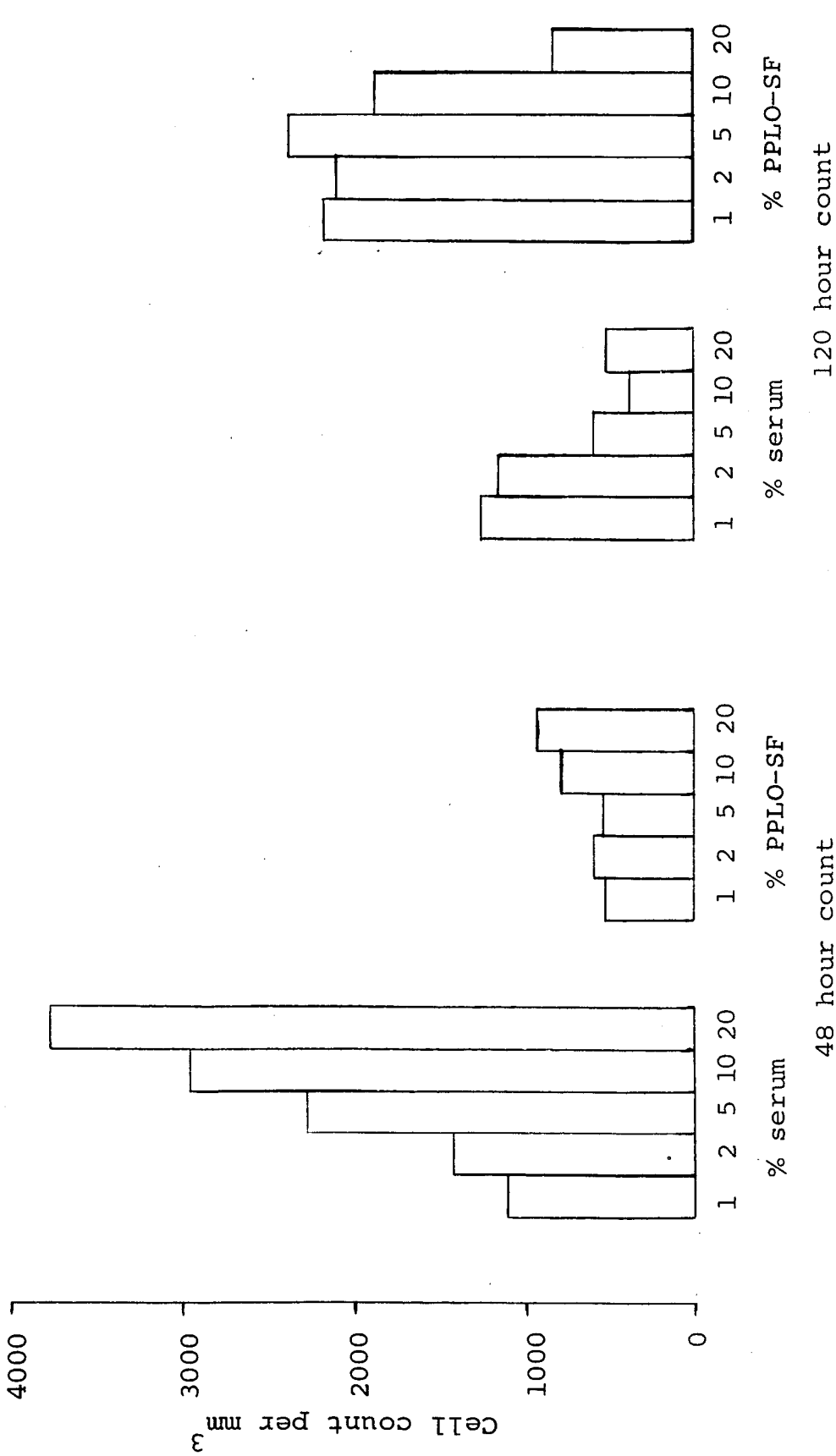


Figure 2. Cell counts of T. foetus strain M in basal medium with horse serum and PPLO serum fraction in varying concentration.

concentration of the PPLO serum fraction. As might be anticipated, an increased concentration of serum in the culture medium gave an increased growth response when counts were made at 48 hours. This was generally true for the PPLO serum fraction but the difference between concentrations was not nearly so marked. Figures 3 and 4 are graphic presentations of the 48 hour data.

Counts at 120 hours (Figure 2) revealed more motile flagellates per mm^3 in each concentration of PPLO serum fraction than in the corresponding concentration of horse serum, and in the three lower concentrations (1, 2, and 5 per cent) the average count at this time was higher than for the corresponding concentration of horse serum at 48 hours.

III. SERUM FRACTIONS PREPARED BY THE ETHANOL FRACTIONATION METHOD AS A REPLACEMENT FOR SERUM.

PPLO serum fraction, which was demonstrated to support multiplication of T. foetus strain M in sustained serial subculture, represents only those components in serum precipitable from 0.62 to 1.0 saturation with ammonium sulfate. It was desired to learn if other serum components might be equally effective, and hence commercially available plasma

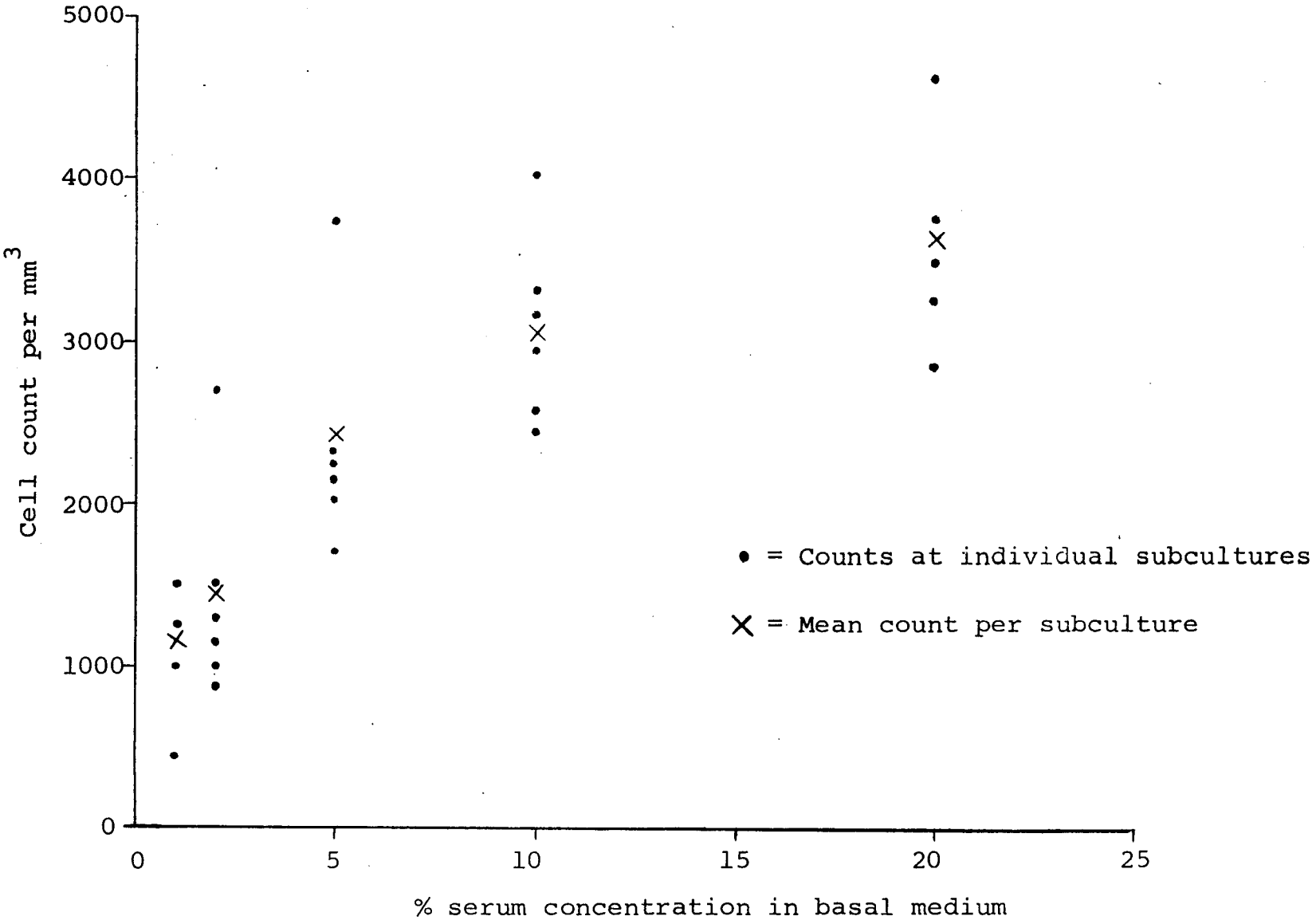


Figure 3. Effect of varying serum concentration on the growth response of T. foetus strain M measured at 48 hours incubation.

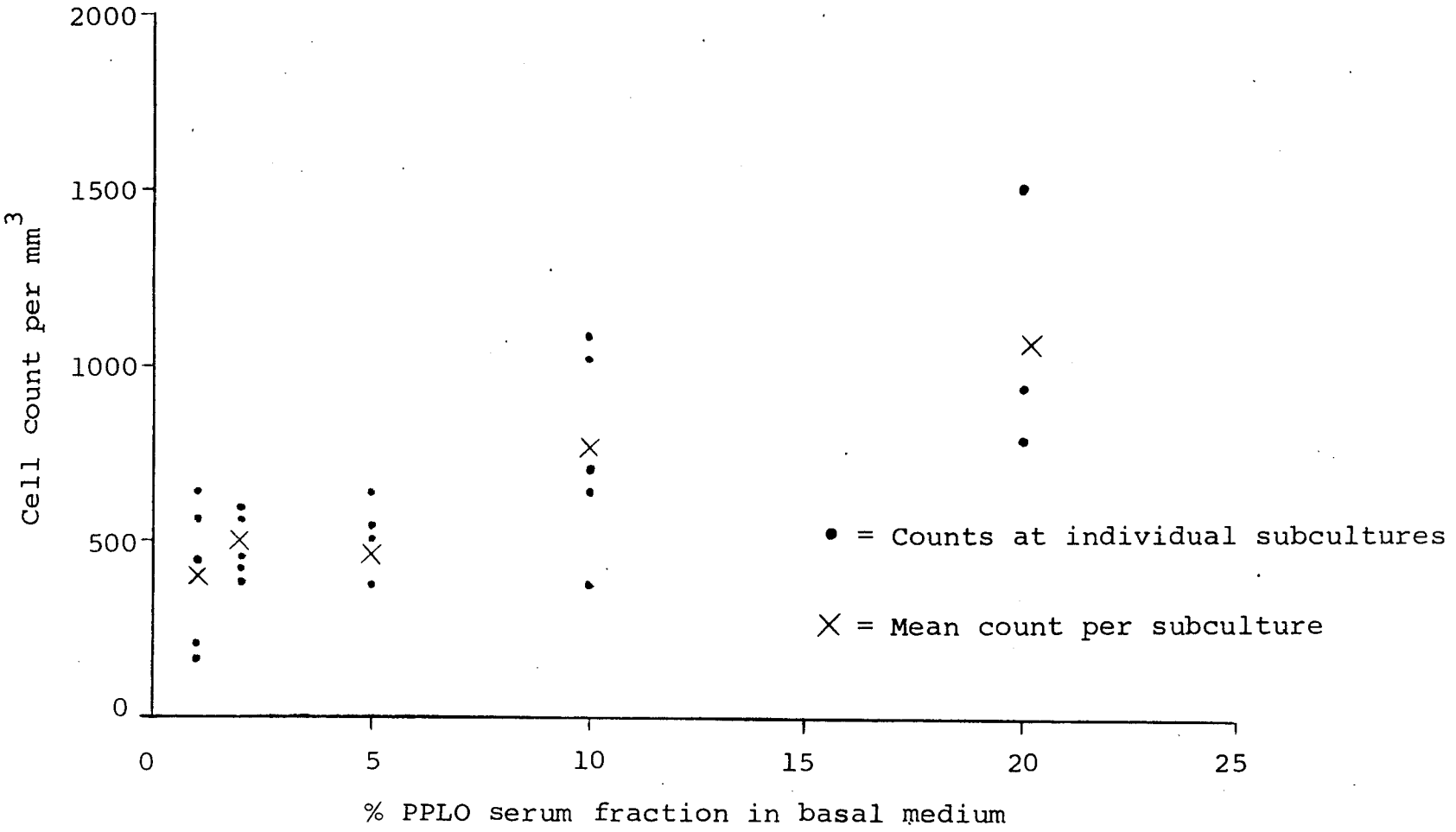


Figure 4. Effect of varying PPLO-serum fraction concentration on the growth response of T. foetus strain M measured at 48 hours incubation.

fractions prepared by the ethanol fractionation method were tried. Results of experiments with these fractions are presented in Figure 5. Serum and PPLO serum fraction controls were run simultaneously with the alcohol fractions and the former were found to follow essentially the same pattern as was observed in earlier experiments. Fractions II + III, IV-1 and IV-4 were found to be able to maintain growth of T. foetus strain M in sustained serial subculture. Counts, however, were considerably lower than those found with either intact serum or PPLO serum fraction. Of the three alcohol fractions, fraction II + III gave the highest mean count at both 48 hours and 120 hours. Fractions V and VI were unable to support sustained growth of T. foetus. Motile trichomonads were noted in the original tubes of medium containing these fractions but first subcultures from these were negative. The use of fractions II + III, IV-1 and IV-4 together in the same medium, each in 5 per cent concentration, failed to demonstrate counts higher than those observed when fraction II + III was used alone. Owing to the extreme insolubility of these fractions they were not tested farther.

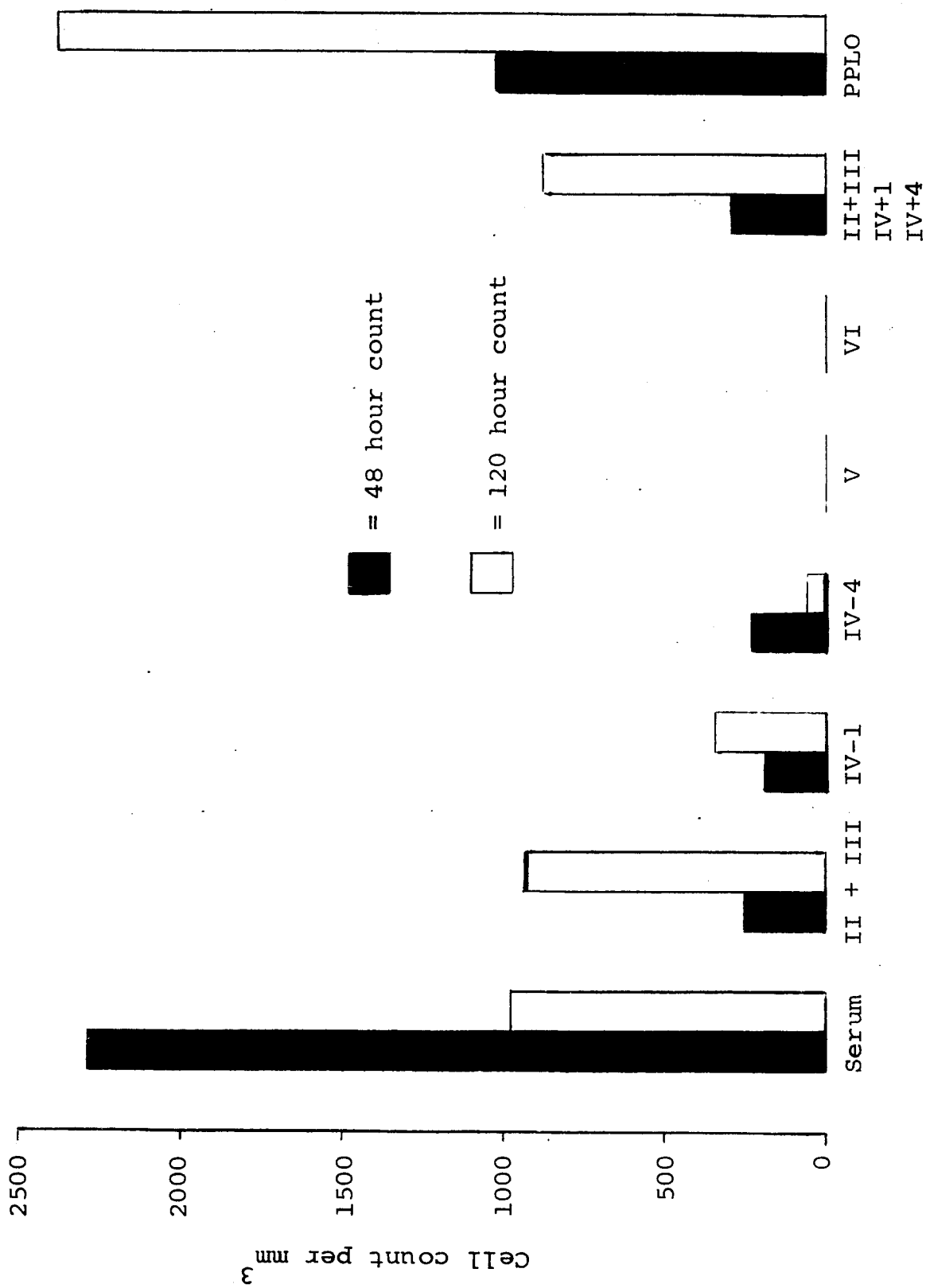


Figure 5. Cell counts of T. foetus strain M in basal medium with serum, PPLO serum fraction and ethanol fractions in 5% concentration.

IV. EXTRACTION OF LYOPHILIZED HORSE SERUM WITH VARIOUS LIPID SOLVENTS.

Lipid extraction of lyophilized horse serum with diethyl ether containing 3.5 per cent alcohol was conducted at room temperature and by refluxing for 24 hours in a Soxhlet extractor. Extraction by the latter method was also performed using acetone, benzene and chloroform. Results of experiments using the insoluble residues in 5 per cent concentration as replacement for intact serum in the culture medium are presented in Figure 6 and the counts by subculture in Table 5. The means of the original seedlings and five subcultures were comparable and analysis of variance (Table 6) showed there was no significant difference among them.

The solvent solubles, after removal of the volatile solvents at reduced pressure, were taken up in 5 per cent human serum albumin. These were tested in 5 per cent concentration for ability to replace intact serum. All were unable to maintain T. foetus strain M on sustained subculture but the acetone soluble fraction did maintain motile trichomonads through two subcultures.

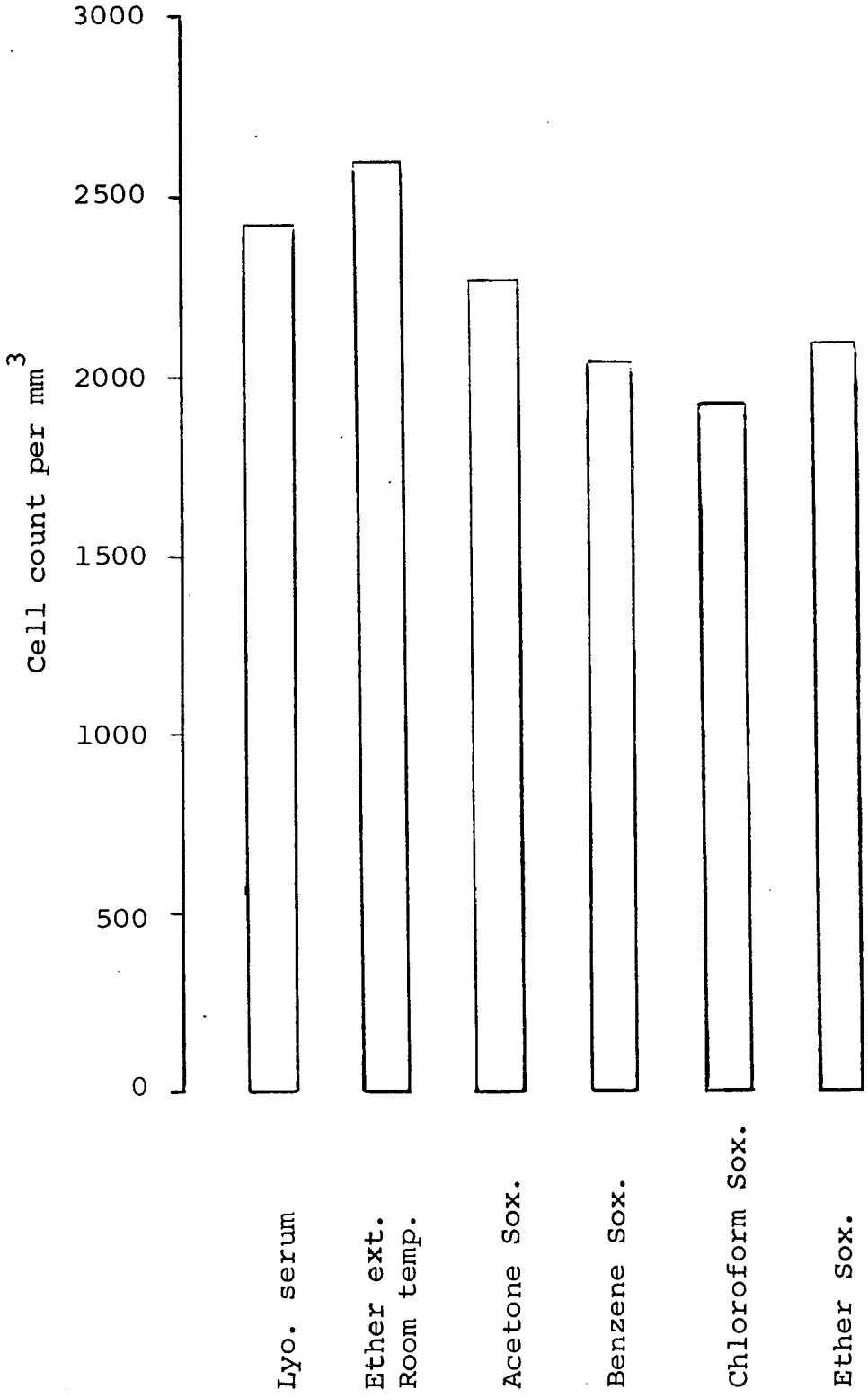


Figure 6. Cell counts of T. foetus strain M in basal medium with 5% reconstituted lyophilized serum and with such serum extracted with different fat solvents. Counts made at 72 hours.

Table 5. Cell counts of T. foetus strain M in Basal Medium with 5% reconstituted lyophilized serum and with such serum extracted with different fat solvents. 72 hour counts. Each count represents the mean of duplicate counts made in duplicate tubes.

Number of Trichomonads per mm^3

Sub-culture No.	Lyo-philized serum	Room T. ether extracted lyo serum	24 hour Soxhlet extraction with: Ace-tone	Ben-zene	Chlor-oform	Ether
Ori-ginal	2325	2050	2500	2475	1800	1550
1	3000	2475	1675	2175	2400	2750
2	2400	2600	2275	1200	1975	1625
3	2250	2250	2125	2225	1875	2425
4	2025	3300	2100	1975	1900	2350
5	2250	2725	2300	2025	1600	1425
Tls.	14250	15400	12975	12075	11550	12125
Mean	2375	2567	2163	2013	1925	2021

Table 6. Analysis of variance of data from fat solvent extraction experiments.

	Degrees of freedom	Sum of squares	Mean sum of squares	F value	Table F values
Total sum of squares	35	6,539,219			
Time sum of squares	5	666,407			
Medium sum of squares	5	1,847,240	367,448	2.28	2.60 2.86
Time x medium	25	4,035,572	161,423		

No significant difference between any of the media.

V. DIALYZED HORSE SERUM AND HORSE SERUM DIALYSATE.

Horse serum was dialyzed for 18 days in the cold against frequent changes of distilled water in order to separate serum constituents into water solubles and water insolubles. These fractions were tested individually and were reconstituted to learn if they could function as a substitute for intact serum. The results of these experiments are shown in Figure 7. Both the water soluble fraction and the water insoluble fraction were able to maintain the test organism in sustained serial subculture. As with PPLO

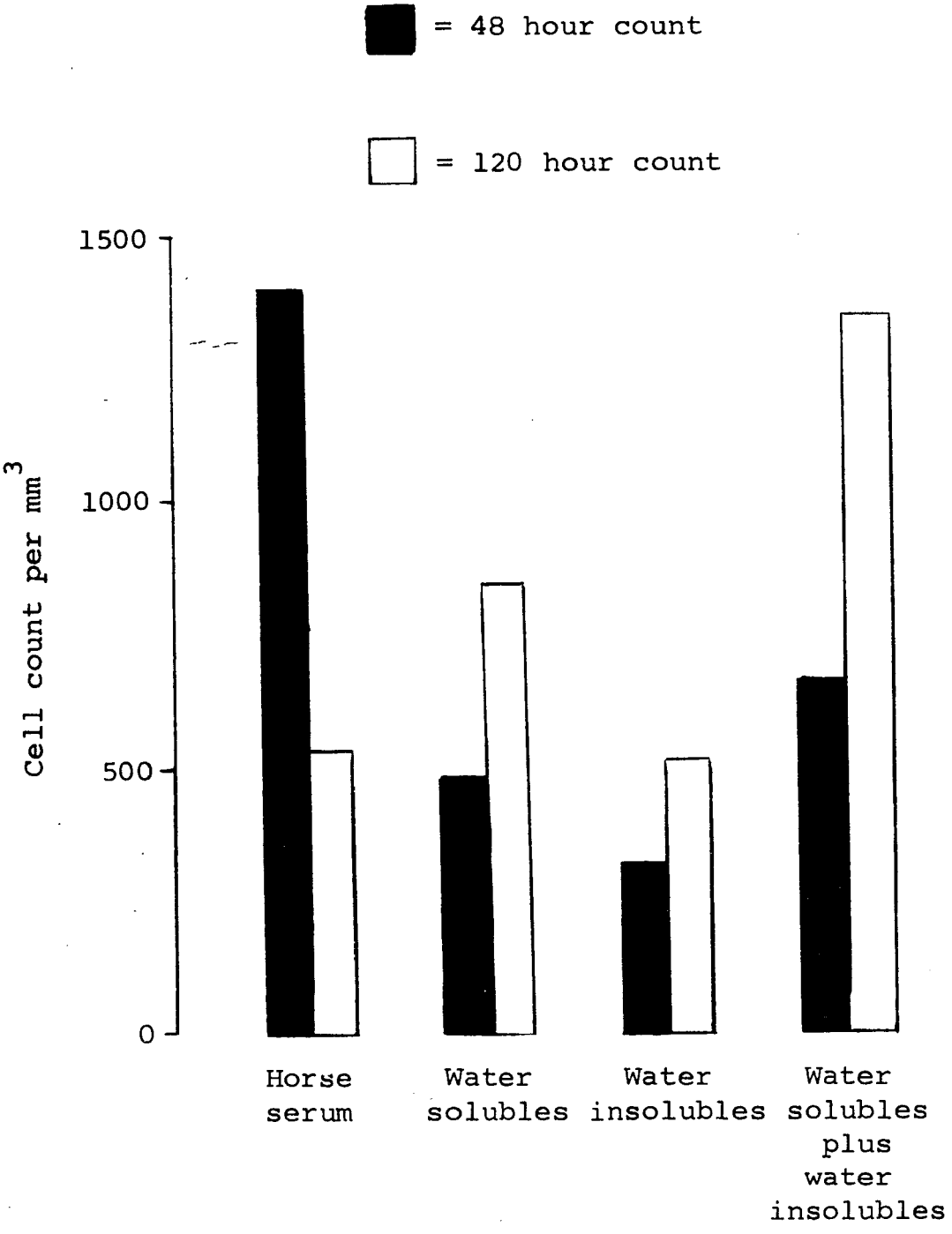


Figure 7. Cell counts of T. foetus strain M in basal medium with 5% horse serum and with water soluble fraction, water insoluble fraction and combined dialysis fractions.

serum fraction the 120 hour count was appreciably higher than the 48 hour count both with the individual fractions and with a recombination of the fractions. The data for both the 48 hour count and the 120 hour count were analyzed statistically (F test). At 48 hours it was found that counts in serum were significantly greater at the 1 per cent level than those in water insolubles, water solubles and recombined fractions. There was no significant difference among water insolubles, water solubles, and recombined fractions at the 5 per cent level. At 120 hours, counts in the recombined fractions were significantly greater at the 5 per cent level than those in intact serum and water insolubles.

Serum dialyzed against distilled water for 18 days is separated into two fractions, water soluble and water insoluble. Both of these were found capable of replacing intact serum for the sustained cultivation of T. foetus strain M although neither of them when used in concentration equivalent to whole serum was as effective as intact serum. Combination of the two fractions did not restore the growth promoting ability observed in 48 hours for intact serum although at 120 hours incubation the number of motile trichomonads in the medium with the combined

fractions was significantly greater than in the medium with intact serum.

It appeared that something was lost on dialysis which might be reclaimable in the dialysate. Accordingly an experiment was conducted in which dialysis was allowed to proceed for 72 hours and the dialysate concentrated to the original serum volume and tested.

Figure 8 shows the results of the experiments comparing growth with intact serum and with horse serum dialyzed 72 hours with counts being made at 48, 72 and 120 hours. The differences seen here compare favorably with those observed between intact horse serum and the recombined fractions obtained when horse serum was dialyzed for 18 days. In each instance the dialyzed horse serum supported growth the pattern of which was essentially the same as that seen when PPLO serum fraction was used. Analysis of variance showed there was a highly significant difference between the intact horse serum and the 72 hour dialyzed horse serum at the 48 and 72 hour counts and no significant difference at the 120 hour count.

Fifty-four hundred ml of horse serum dialysate, collected over a period of 72 hours, were lyophilized and later reconstituted with distilled water to the original

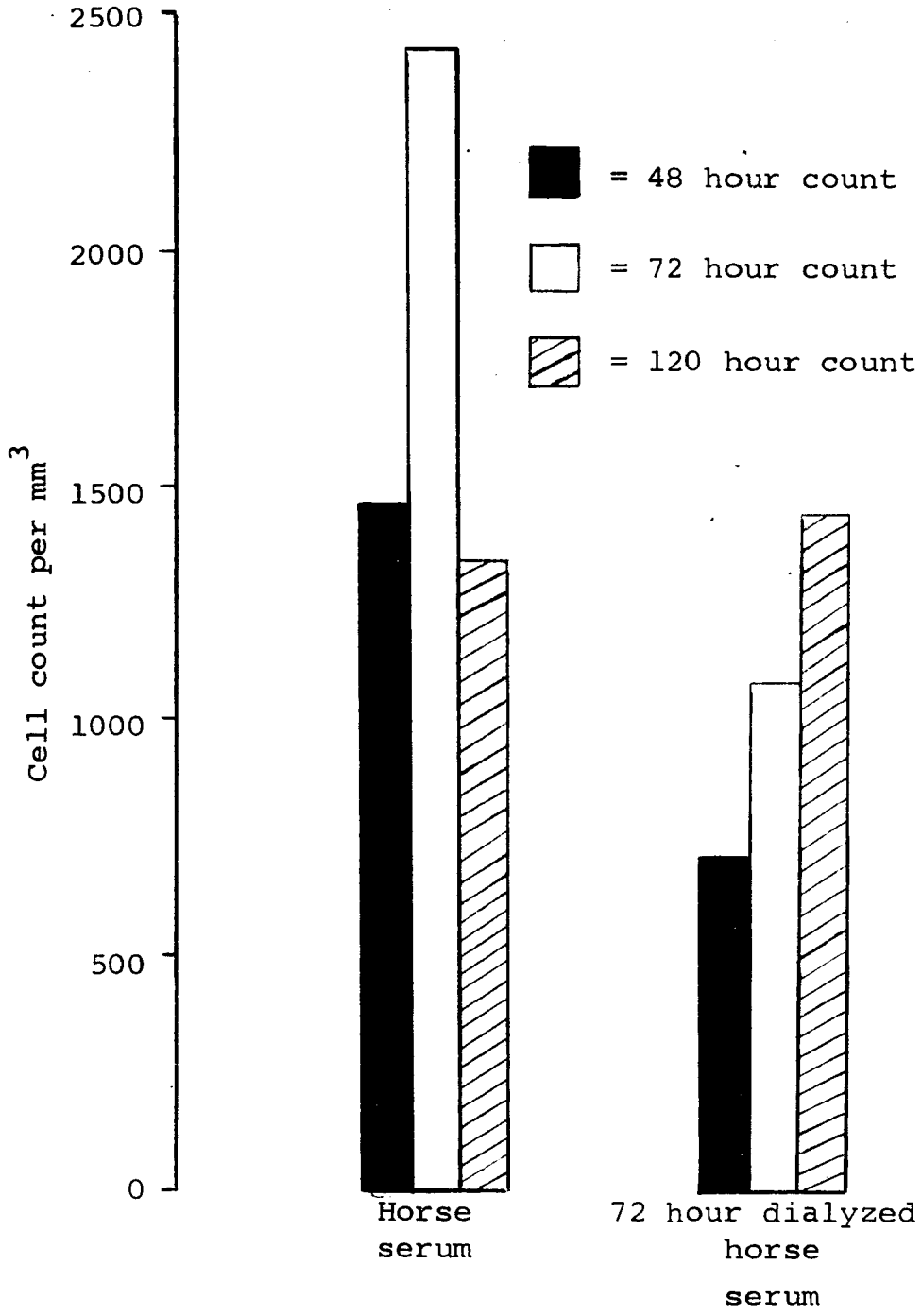


Figure 8. Cell counts of T. foetus strain M in basal medium with 5% horse serum and with horse serum dialyzed for 72 hours.

volume of the serum, 200 ml, from which it was collected. This represented nine 600 ml volumes of dialysate which were collected and pooled at approximately eight hour intervals over the course of the 72 hour dialyzing period. This was tested for its ability to support multiplication of T. foetus strain M in concentrations of 2, 5, 10 and 20 per cent and in combination with 72 hour dialyzed horse serum at 5 per cent concentration. The results of these trials are shown in Figures 9 and 10. The dialysate was capable of maintaining T. foetus even at a concentration of 2 per cent in the culture medium although the mean count per transfer was only 159 cells per mm^3 at 72 hours incubation. The count was 157 at a concentration of 5 per cent, 396 at 10 per cent and 636 at 20 per cent. Plotting of the individual subculture counts around the mean, Figure 9, indicated the great degree of variability that was encountered from transfer to transfer using the dialysate as a serum substitute, especially at 10 and 20 per cent concentrations.

The combination of 5 per cent dialysate and 5 per cent dialyzed horse serum gave an average count per transfer of 1429 cells per mm^3 as compared with 1088 for 5 per cent dialyzed serum alone. Analysis of variance of the data

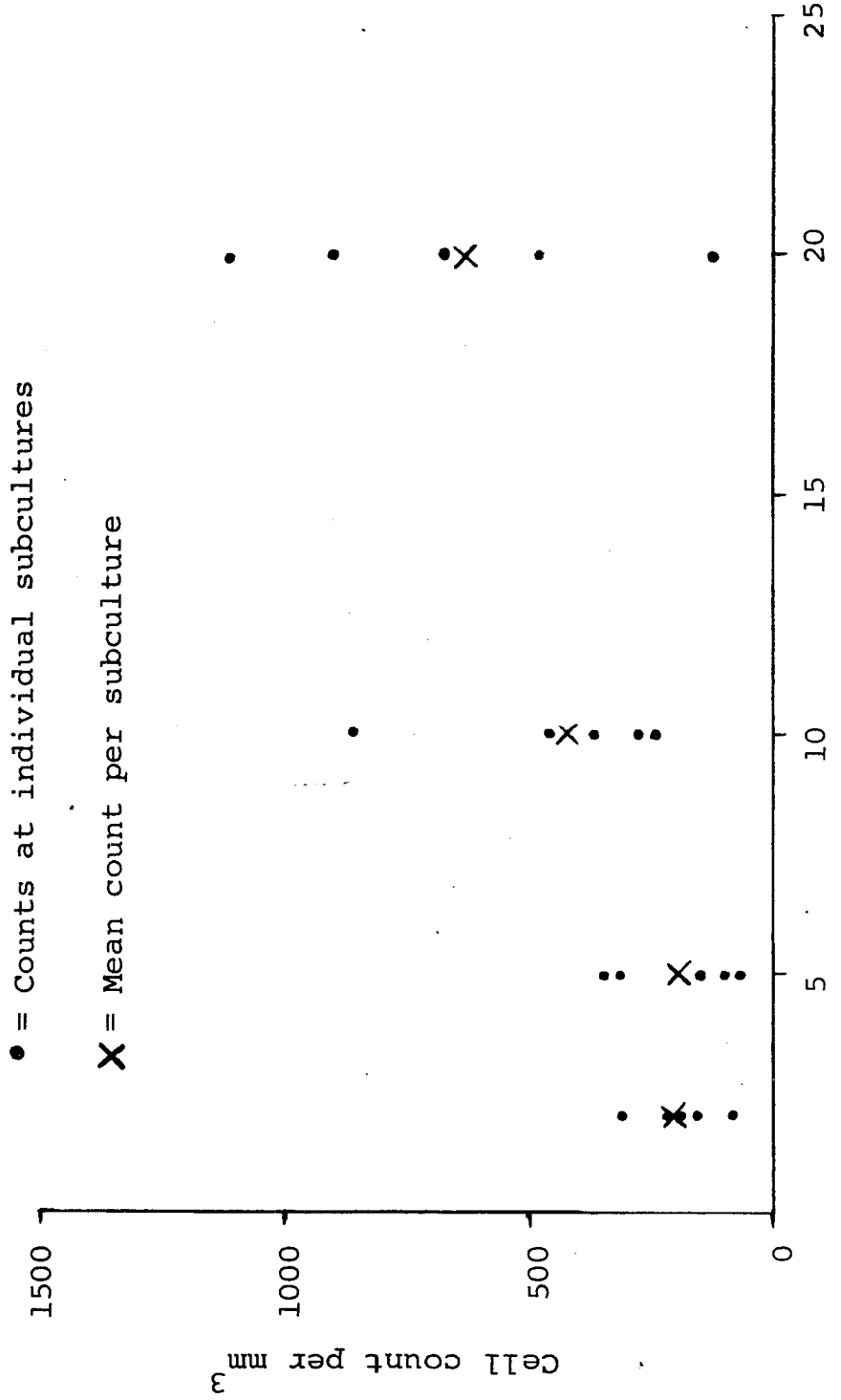


Figure 9. Effect of varying concentrations of 72 hour serum dialysate on the growth response of T. foetus strain M at 72 hours.

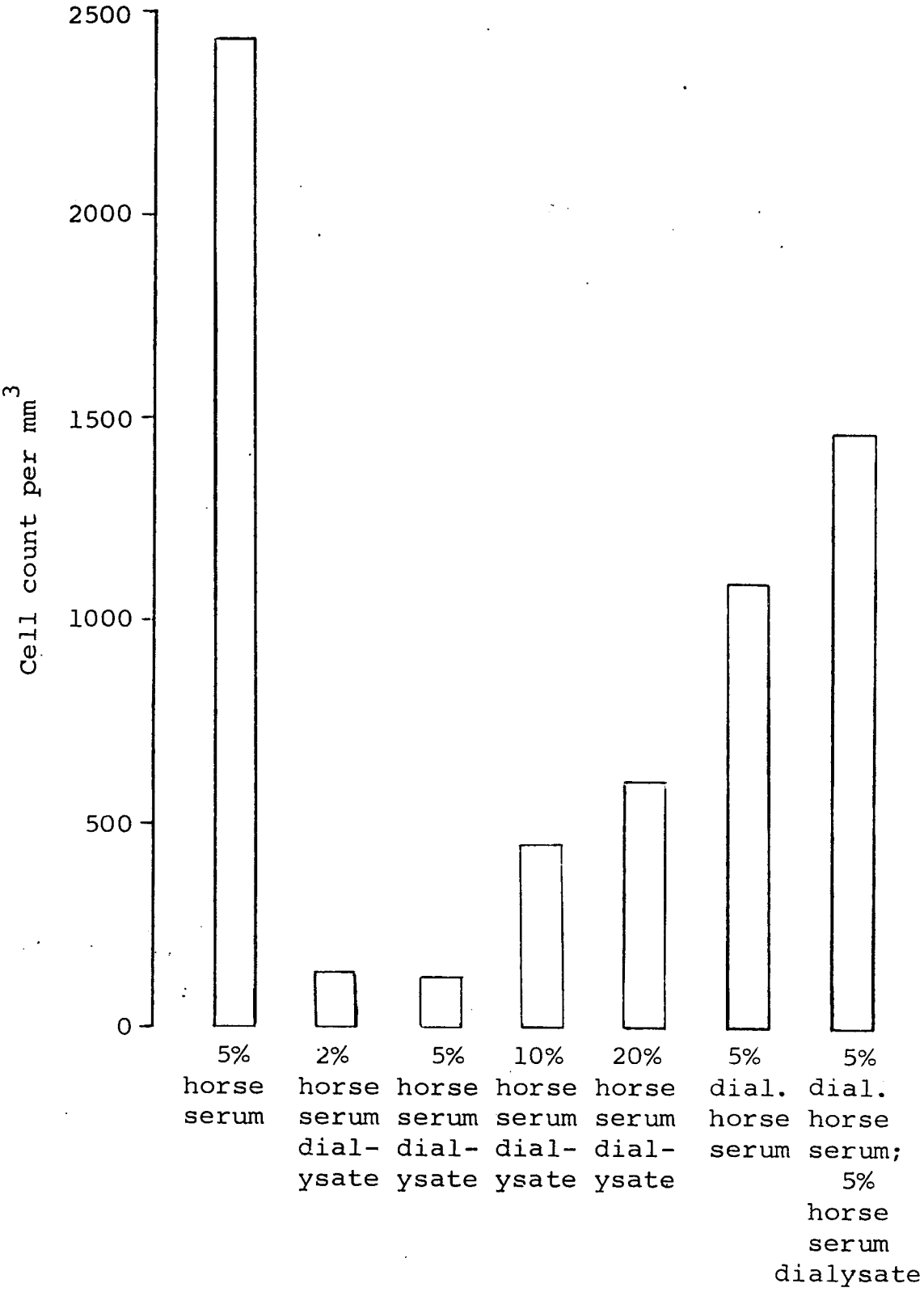


Figure 10. 72 hour cell counts of T. foetus strain M in basal medium with serum, dialyzed horse serum and serum dialysate as indicated.

from this comparison showed there was a significant difference at the 5 per cent level.

VI. AMMONIUM SULFATE FRACTIONS AS SUBSTITUTES FOR INTACT SERUM

Six fractions were obtained and were numbered 1 through 6. One through 5 were collected as precipitates which were obtained at ammonium sulfate molar concentrations of 1.39, 1.64, 2.05, 2.57 and 2.80 respectively. Fraction 6 was the material that was soluble in these increasing concentrations of ammonium sulfate.

These fractions were tested in concentrations of 2, 5, 10 and 20 per cent. Fraction 2 was found incapable of maintaining T. foetus strain M in serial subculture, the trichomonads not surviving beyond the second subculture at concentrations less than 10 per cent. Figure 11 shows the results of these experiments. Fractions 1, 3 and 6 gave increasingly higher counts after 72 hours incubation with an increase in concentration of the fraction. Fraction 5 gave the highest mean count at the 5 per cent concentration level, the level of serum concentration commonly used in culture media. Of the six fractions, fraction 5 showed the least difference in mean cell count with changing concentration.

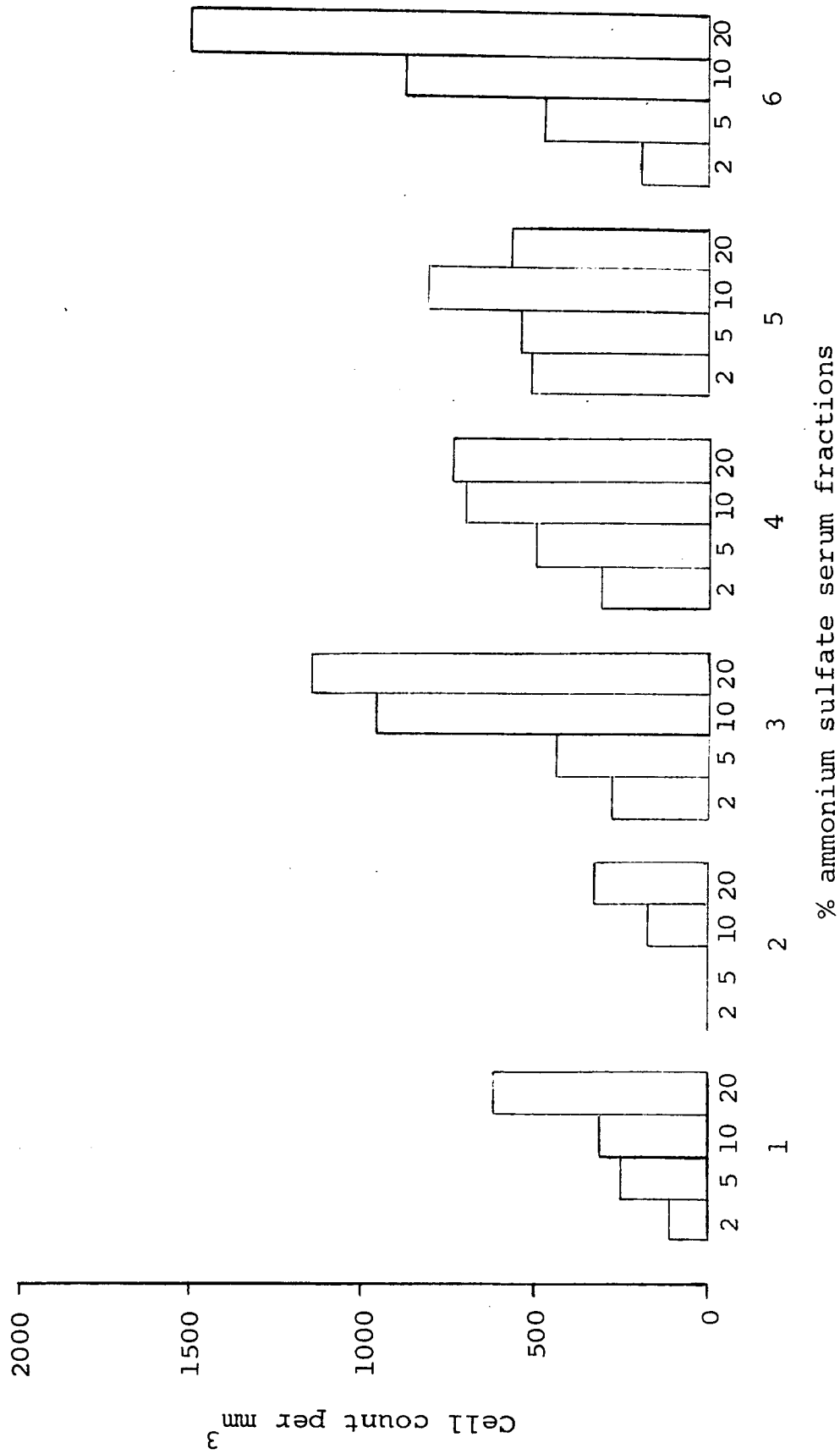


Figure 11. 72 hour cell counts of T. foetus strain M in basal medium with ammonium sulfate serum fractions in varying concentrations.

Fractions 1, 3, 4, 5 and 6 were added to the basal medium in 5 per cent concentration and comparisons made of the cell counts at 48 and 120 hours incubation. The results of these tests are shown in Figure 12. Average counts shown for 72 hours incubation in this figure were from the data shown in Figure 11. At this concentration fraction 5 gave higher mean counts than any of the other fractions tested. Analysis of variance showed there was no significant differences among the counts at 48 hours. At 120 hours, counts in fraction 5 were significantly greater at the 1 per cent level than counts in fractions 1, 3, 4 and 6; counts in fractions 4 and 6 were significantly greater at the 1 per cent level than counts in fraction 1; and counts in fraction 4 were significantly greater at the 5 per cent level than counts in fraction 3. Thus fraction 5, over the 120 hours incubation period, was the fraction maintaining the highest mean count.

VII. THE EFFECT OF ASCORBIC ACID AND PANTOTHENIC ACID ALONE AND COMBINED ON THE GROWTH RESPONSE OF *T. FOETUS* IN BASAL MEDIUM WITH AMMONIUM SULFATE SERUM FRACTIONS.

Kupferberg *et al.* (1948) had shown a requirement for pantothenic acid by *T. vaginalis* when the serum concentration

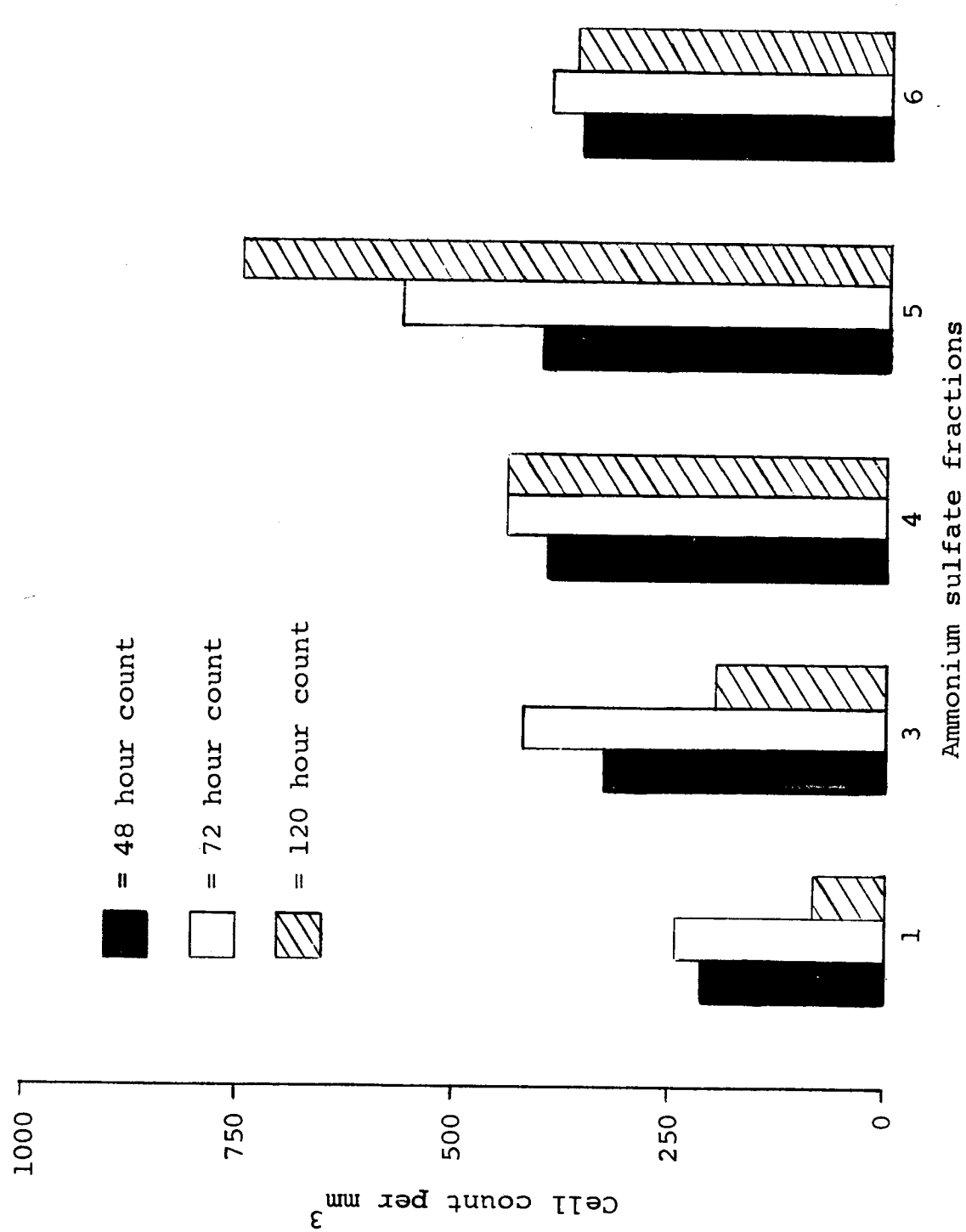


Figure 12. Cell counts of T. foetus strain M in basal medium with ammonium sulfate fractions 1, 3, 4, 5 and 6 in 5% concentration.

was reduced to 0.5 per cent. Rieck (1953) and Cailleau (1938a) reported the effect of ascorbic acid on the growth of T. foetus. These substances were tried alone and combined with five fractions (1, 3, 4, 5 and 6) of serum obtained by ammonium sulfate fractionation. The results are shown in Figure 13. Ascorbic acid was found to have a marked effect when used with fractions 4 and 5. Pantothenic acid had only a very moderate influence when used with fraction 5. Analysis of variance was done for each of the fractions. Counts in fractions 4, 5 and 6 containing ascorbic acid were significantly higher at the 1 per cent level than in the same fractions without ascorbic acid or with pantothenic acid. Counts in fractions 4, 5 and 6 containing both ascorbic acid and pantothenic acid were no higher than with ascorbic acid alone in each of the fractions.

Neither ascorbic acid nor pantothenic acid alone or combined would support T. foetus without the presence of serum or one of the serum fractions.

Ammonium sulfate fraction 5 in 10 per cent concentration supplemented with ascorbic acid in a concentration of 1 mg per ml in the basal medium was tested for its ability to support T. vaginalis and other strains of T. foetus.

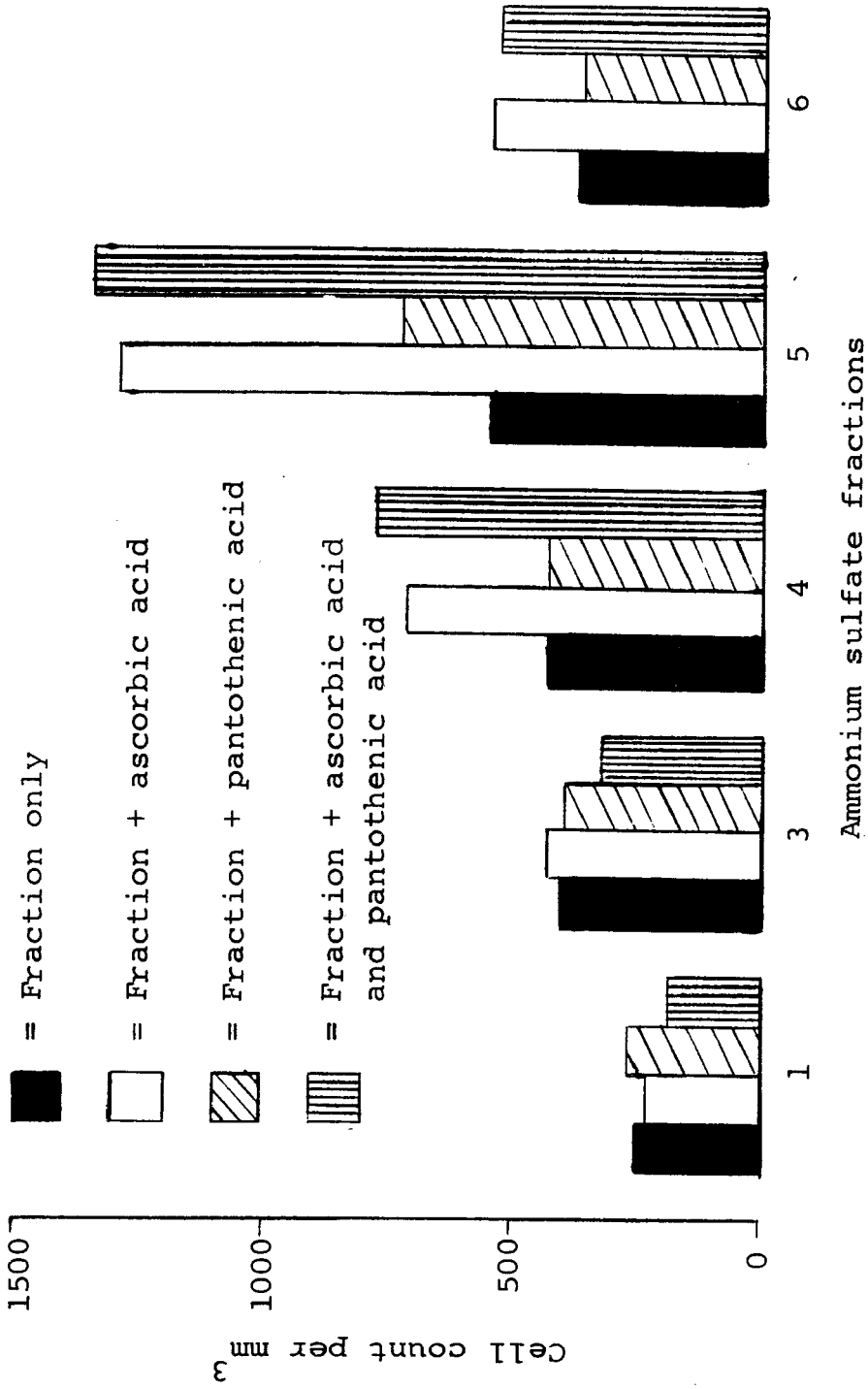


Figure 13. 72 hour cell counts of T. foetus strain M in basal medium with ammonium sulfate fractions alone and with ascorbic acid and pantothenic acid individually and combined.

Accordingly one strain of the former and two strains of the latter, designated strains L and UT, were tried. Trichomonas vaginalis could not be maintained in sustained serial subculture in this medium, the trichomonads disappearing on the first subculture. Three different attempts with this species gave uniformly negative results. Comparisons of growth response of the three strains of T. foetus with 5 per cent serum and with 10 per cent fraction 5 and ascorbic acid in the basal medium are shown in Figures 14 and 15.

Analysis of variance of the data represented in Figure 14 showed there to be no significant difference between media when growth response was measured using a given strain. Strains M, L and UT respectively grew as well in the medium containing fraction 5 and ascorbic acid when counted at 72 hours post inoculation as they did in the medium containing intact serum.

Analysis of variance of the data represented in Figure 15, which is a comparison of the growth response of the three strains in each of the media, showed strain M counts to be significantly higher at the 1 per cent level than both strain L and strain UT counts when grown in medium containing serum. When grown in medium containing 10 per

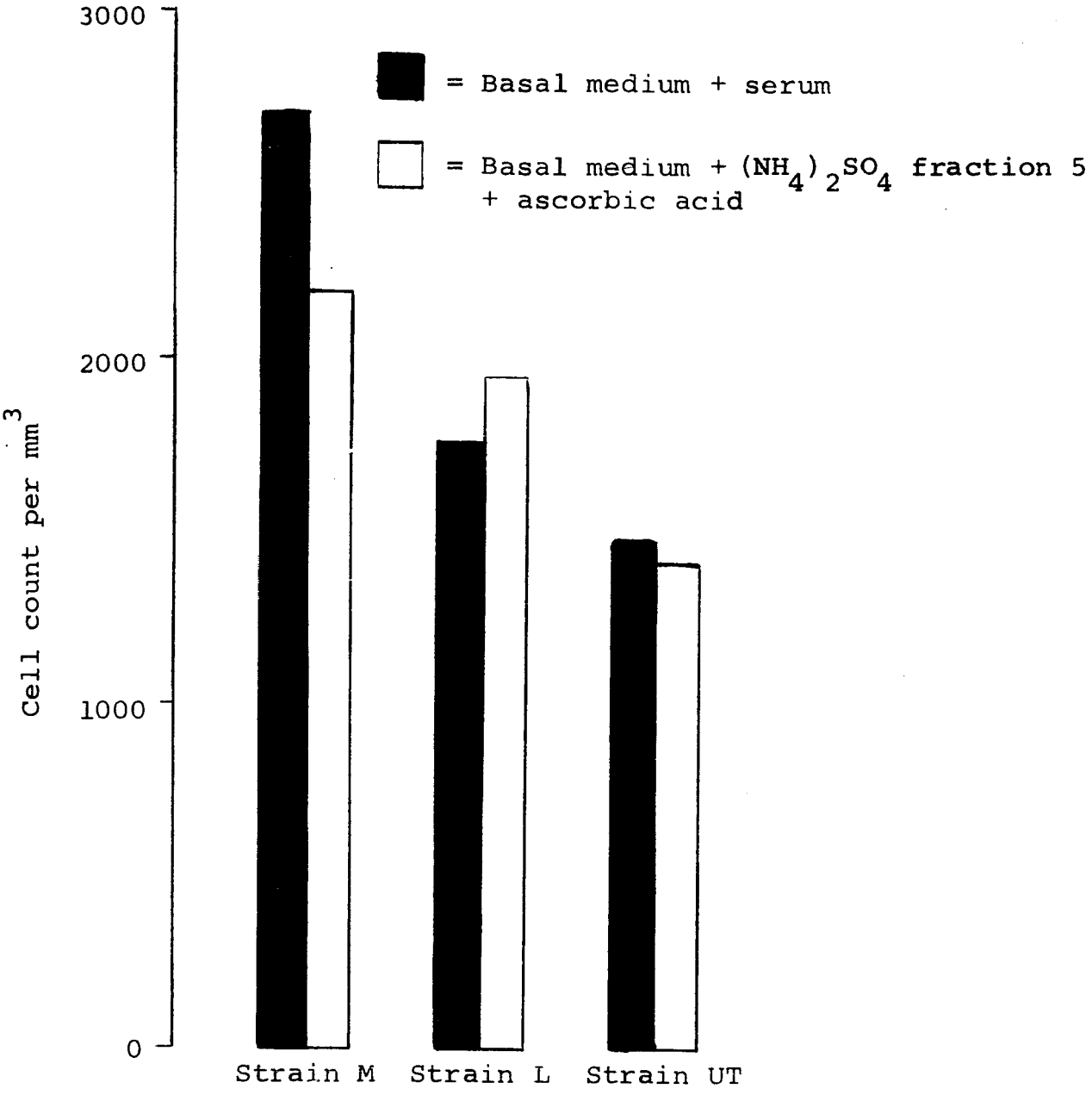


Figure 14. 72 hour cell counts of T. foetus strains M, L and UT in basal medium with 5% serum and with 10% ammonium sulfate fraction 5 with 0.1% ascorbic acid.

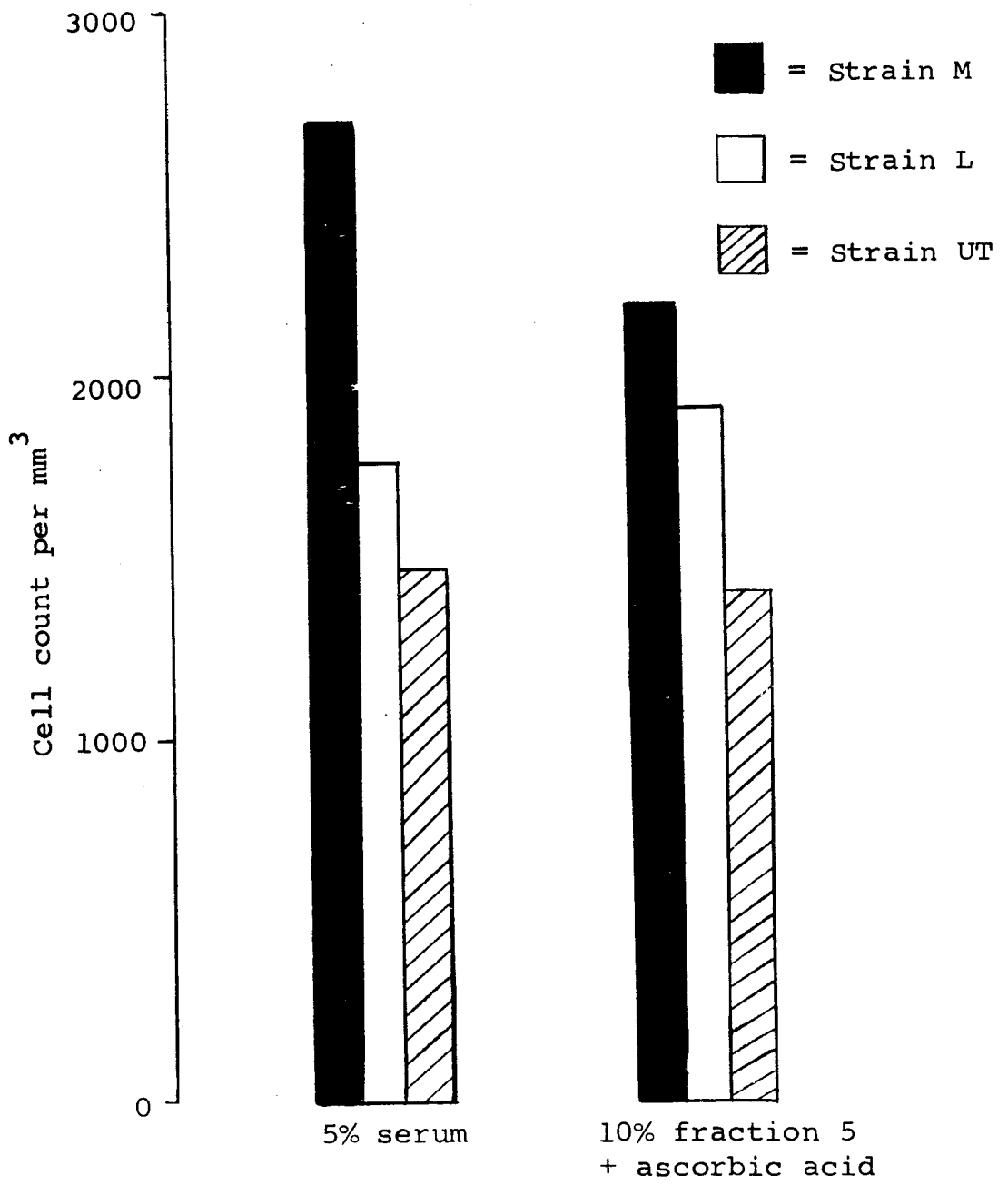


Figure 15. 72 hour cell counts of T. foetus strains M, L, and UT in basal medium with 5% serum and with 10% ammonium sulfate fraction 5 with 0.1% ascorbic acid.

cent fraction 5 with ascorbic acid strain M counts were significantly higher at the 1 per cent level than strain UT counts, significantly higher than strain L counts at the 5 per cent level and strain L counts were significantly higher than strain UT counts at the 5 per cent level.

Thus it is seen there is a difference among strains regardless of which medium they are in but there is no difference in the growth response of a given strain when compared in the two different media. In other words the difference is among strains and not between media.

VIII. THE INFLUENCE OF CYSTEINE IN THE CULTURE MEDIUM ON GROWTH RESPONSE.

Cysteine has been a constituent of most culture media for the pathogenic trichomonads since the initial work with CPLM medium by Johnson and Trussell (1943). It has not been demonstrated to be an essential nutrient but has been thought to function as a reducing agent. The effect of deleting this compound from culture media for the growth of T. vaginalis and three strains of T. foetus is shown in Figure 16. Analysis of variance of these data showed there was no significant difference between

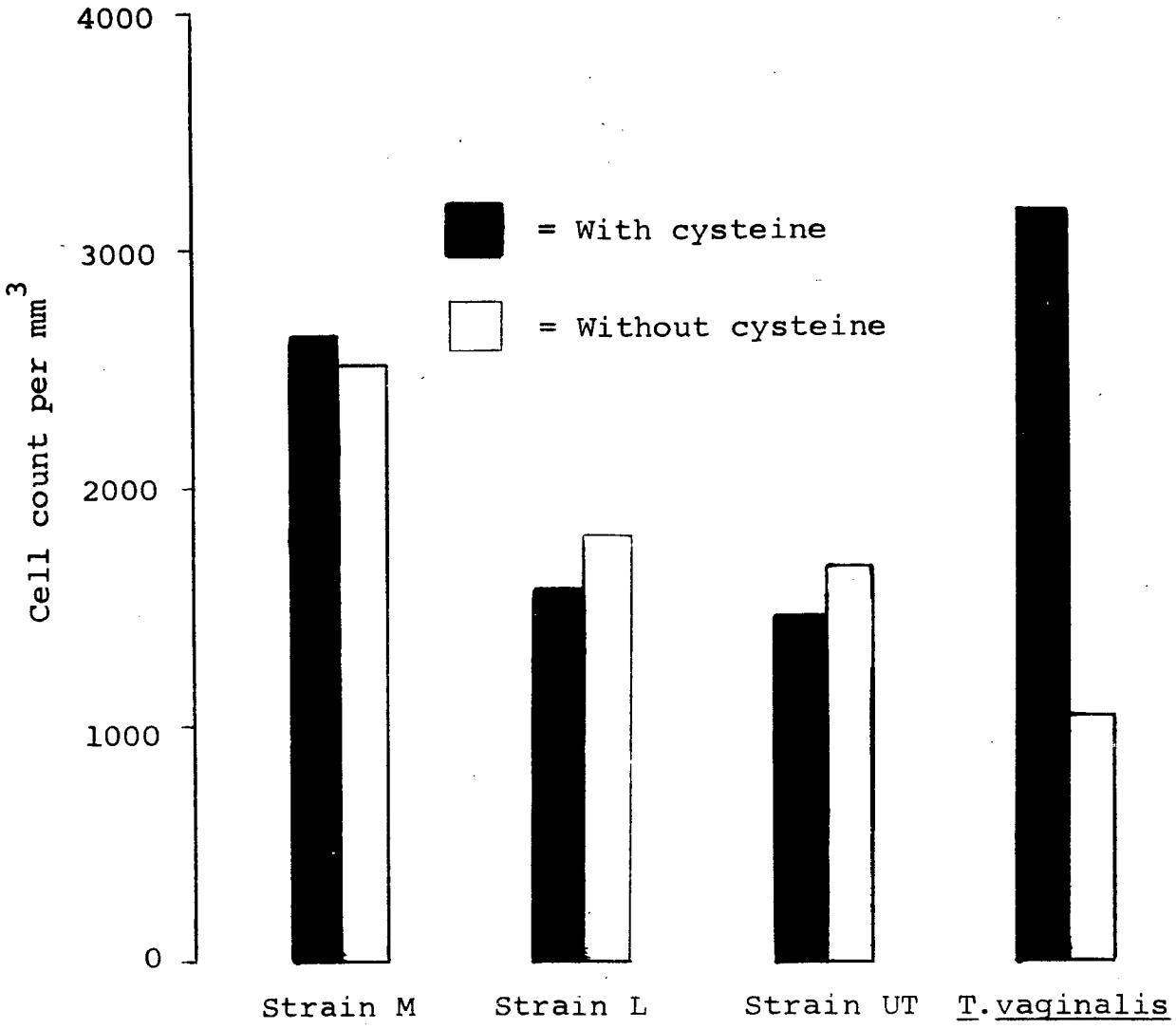


Figure 16. 72 hour cell counts of T. foetus strains M L, and UT and T. vaginalis grown in basal medium including 5% serum, with and without cysteine.

counts of each strain of T. foetus when grown in media with and without cysteine. On the other hand, counts of T. vaginalis were significantly higher at the 1 per cent level in the medium containing cysteine than in the same medium but without cysteine.

It is evident that cysteine can be deleted from the basal medium for the growth of T. foetus where serum is used in 5 per cent concentration and where incubation is under reduced oxygen tension. Deleting this compound from medium for the cultivation of T. vaginalis under the same conditions allowed the trichomonad to be maintained in serial subculture but at populations significantly below those obtained when cysteine was included in the medium.

IX. THE USE OF HEATED SERUM IN THE BASAL CULTURE MEDIUM.

Experiments with heated serum were conducted in two ways. In one experiment the serum was heated to 100 C for 20 minutes and the fluids removed from the coagulum prior to cooling. This fluid was used in 5 per cent concentration in the basal medium in place of intact serum and counts were made at 48 and 120 hours. The results are shown in Figure 17. This liquid was found capable of maintaining T. foetus

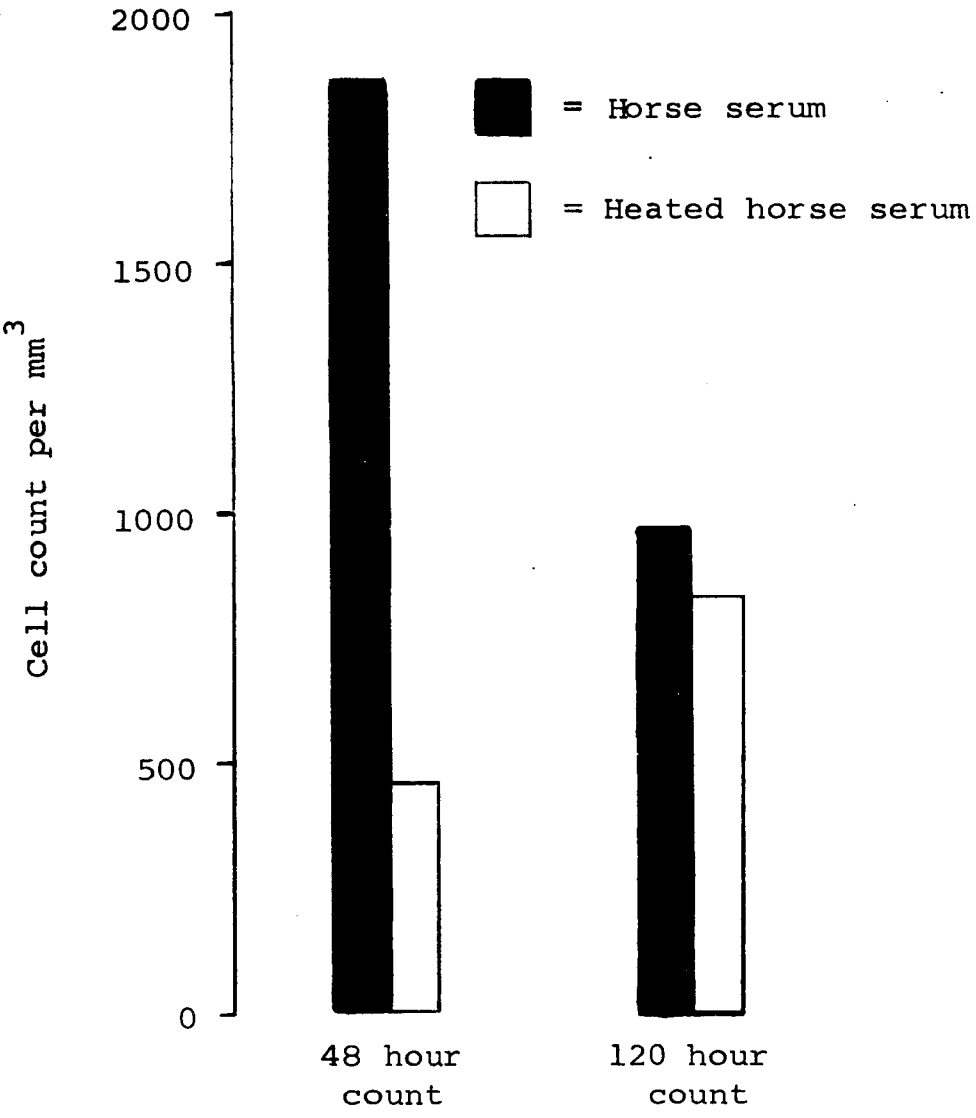


Figure 17. Cell counts of T. foetus strain M in basal medium with unheated serum and with serum heated to 100 C for 20 minutes.

in serial subculture although to a level much lower than intact serum, especially when counted 48 hours post inoculation. Analysis of variance of these data showed a highly significant difference at the 48 hour count but no significant difference at the 120 hour count.

In a second experiment serum was added to the culture medium in 5 per cent concentration and this was autoclaved at 121 C for 10 minutes. Ascorbic acid in 0.1 per cent concentration was added to the heated serum and also to the unheated serum controls. Results are shown in Figure 18. Analysis of variance showed that serum heated as indicated maintained T. foetus strain M in serial subculture although at a level significantly lower than unheated serum. The use of ascorbic acid in 0.1 per cent concentration did not significantly affect the trichomonad counts when used with either heated or unheated serum.

X. EFFECT OF PROTEIN PRECIPITATION WITH ETHANOL.

Proteins were precipitated from serum by the use of three volumes of 95 per cent ethanol. Alcohol soluble material in 5 per cent concentration was tested for its ability to support multiplication after removal of the alcohol by evaporation through dialyzing tubing at 4 C.

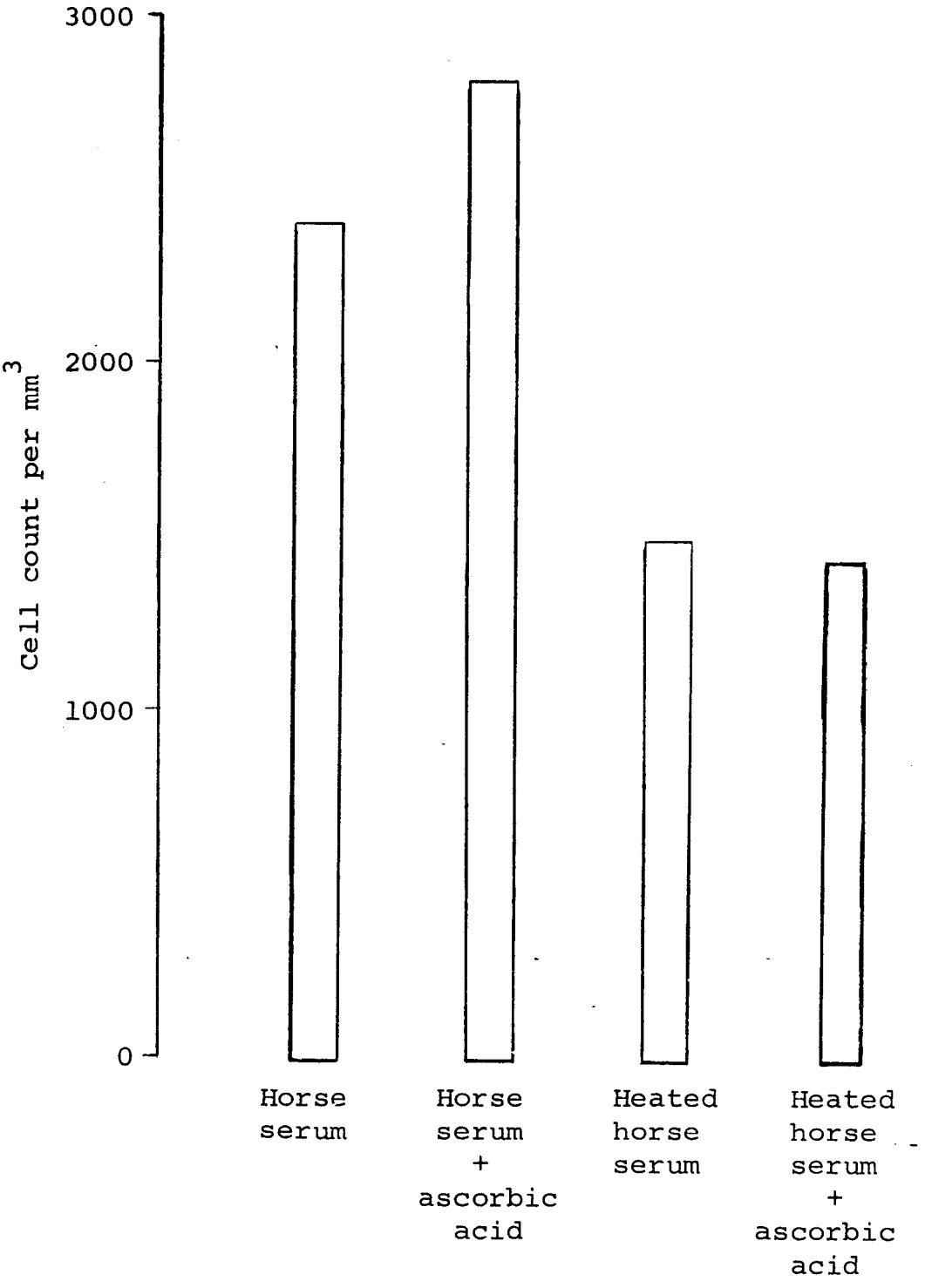


Figure 18. 72 hour cell counts of T. foetus strain M in basal medium with unheated serum and with serum autoclaved at 121 C for 10 minutes both alone and with 0.1% ascorbic acid.

Trichomonad growth was not maintained beyond the original seeding. To learn if some of the alcohol may have remained in the alcohol soluble preparation 5 per cent was added to the basal medium containing 5 per cent serum. The mean count per transfer for the original seeding and five subcultures was 2567 cells per mm^3 as compared with 2375 cells per mm^3 with serum only. This difference was not statistically significant.

XI. OTHER SUBSTANCES TESTED

a. Two per cent hemoglobin in concentrations of 1.25, 2.5, 5.0, and 10 per cent was tried as serum replacement. Uniform negative results were obtained, growth not being maintained beyond the original seeding.

b. Cholesterol and Tween 80 were tested for their ability to replace serum in the basal medium. Sanders (1957) reported these substances capable of replacing serum in a much more complex medium than the basal medium used herein. Concentrations of 0.25, 0.50, 1.00, 2.50, and 5.00 mg per cent of cholesterol were tried with Tween 80 concentrations of 0.5, 1.0, and 2.0 mg per cent. All combinations were incapable of maintaining T. foetus strain M beyond the original seeding.

XII. FRACTION IDENTIFICATION

a. Tests for Carbohydrate, Cholesterol and Protein. Table 7 shows the results of the tests for the presence of carbohydrate, cholesterol, and protein. All materials tested for carbohydrate by the Molisch Test were considered positive and only ammonium sulfate fraction 4 gave anything other than a clear cut positive reaction. This fraction also gave a doubtful or very weakly positive reaction for sterols by the Salkowski Test and also demonstrated a very fine precipitate when heated to 100 C for 20 minutes. Fraction 5, the fraction which when used with ascorbic acid gave the highest trichomonad counts of all the fractions tested, gave positive tests for carbohydrate, sterol and protein. Dialyzed serum and serum dialysate were negative for cholesterol but were positive for carbohydrate and protein. The fact that precipitation occurred on heating the serum dialysate would indicate a leakage of protein through the dialyzing bag, something which should not occur if the apparatus were functioning properly. Heated serum solubles gave positive reactions only for carbohydrate and protein.

Lyophilized serum extracted with ether at room temperature gave a positive sterol reaction by the Salkowski

Table 7. Qualitative test results for carbohydrate, cholesterol and protein in serum fractions used to replace intact serum in the basal medium.

Fraction designation	Molisch test for CHO	Cholesterol		Protein			
		Liebermann	Salkowski	100 C	Biuret	Heller's	TCA
Serum control	++++	+	+	+	+	+	+
Cholesterol control	0	+	+	0	0	0	0
18 day dial. sols.	++++	-	-	+	+	0	0
18 day dial. ppt.	++	-	-	+	+	0	0
72 hr. dialysate	++	-	-	+	+	+	+
$(NH_4)_2SO_4$-1	++	-	±	+	+	0	0
$(NH_4)_2SO_4$-3	++++	-	±	+	+	0	0
$(NH_4)_2SO_4$-4	±	-	±	±	+	+	+
$(NH_4)_2SO_4$-5	+	-	+	+	+	0	0
$(NH_4)_2SO_4$-6	+	-	+	+	+	0	0
Heated serum sols.	++	-	-	+	+	+	+
Alc. sols.	-	-	-	-	-	-	-
Eth. Ext. Rm.T.	0	-	+	0	0	0	0
Acet. Ext. Sox.	0	-	-	0	0	0	0
Benz. Ext. Sox.	0	-	-	0	0	0	0
Chlor. Ext. Sox.	0	-	-	0	0	0	0
Ether Ext. Sox.	0	-	-	0	0	0	0
Lyo. Serum	0	+	+	0	0	0	0
Acet. Sol. + BA	0	+	+	0	0	0	0
Benz. Sol. + BA	0	-	-	0	0	0	0
Chloro. Sol. + BA	0	+	+	0	0	0	0
Ether Sol. + BA	0	+	±	0	0	0	0
BA control	0	-	-	0	0	0	0

+ = positive; - = negative; 0 = not tested.

method but was negative by the Lieberman method. The two sterol tests were in agreement on the lyophilized serum samples which were Soxhlet extracted using acetone, benzene, chloroform or ether. All Soxhlet extracted serum samples were negative for sterols. Solubles extracted by refluxing with organic solvents, with the exception of that sample extracted with benzene, when taken up in 5 per cent bovine albumin were sterol positive. Benzene solubles were negative by both sterol tests. The bovine albumin control gave a negative reaction by both the Lieberman method and the Salkowski method.

b. Ultracentrifugation Analysis.

Ultracentrifugation was performed on Difco's PPLO serum fraction and on fraction 5 obtained by ammonium sulfate fractionation. Figures 19 and 20 show the results of these respective trials. PPLO serum fraction presented a picture of homogeneity whereas ammonium sulfate fraction 5 was found to contain two major components. Of these one was seen to be appreciably heavier than the other since it sedimented at a much faster rate. In fact, it had traveled the length of the analytical cell and was out of the photographic field by the end of 48 minutes exposure in the centrifugal field. The second component of this fraction

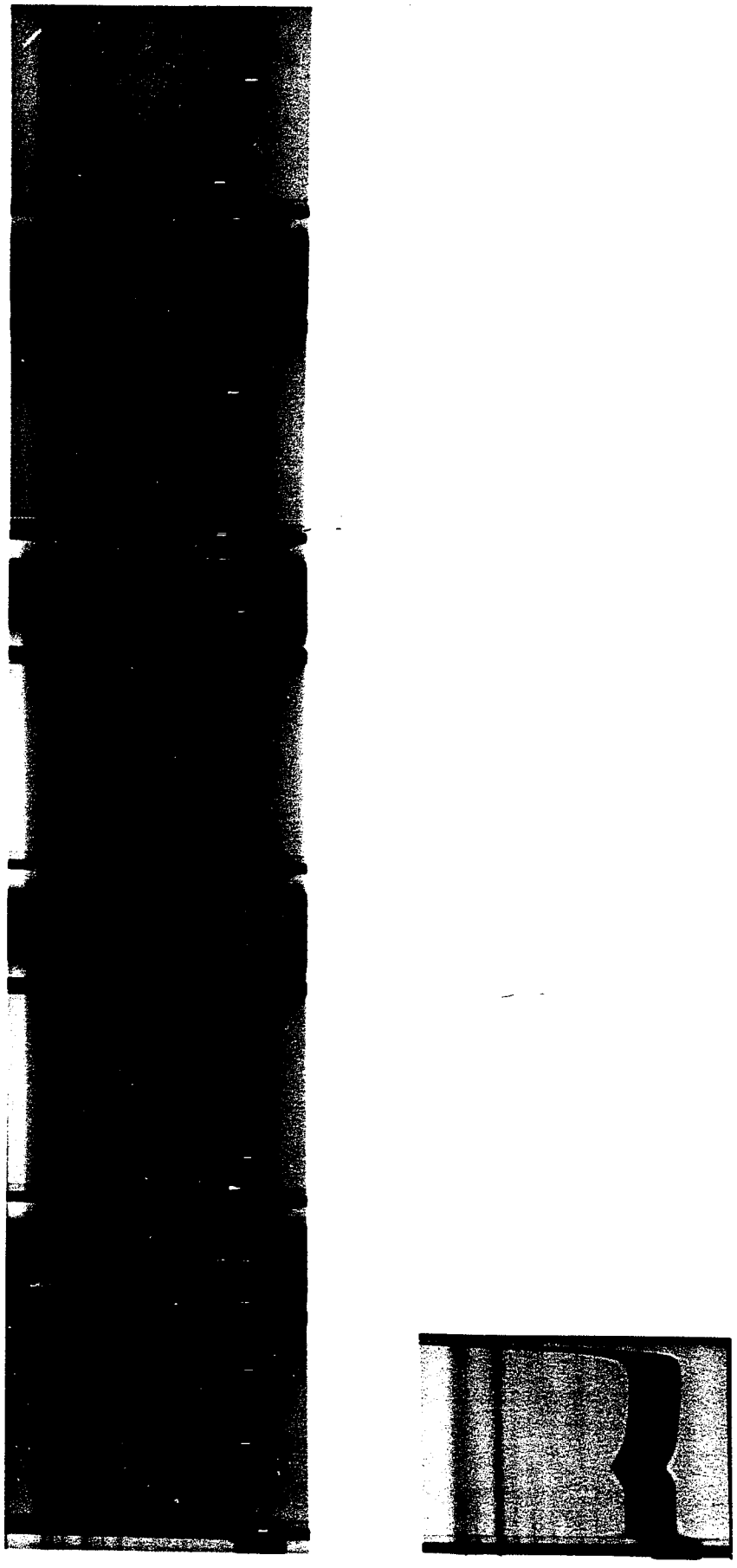


Figure 19. Ultracentrifuge analysis of PPLO serum fraction. 59,780 rpm, voltage 80, 6 sec. exposure at 16 minute intervals.

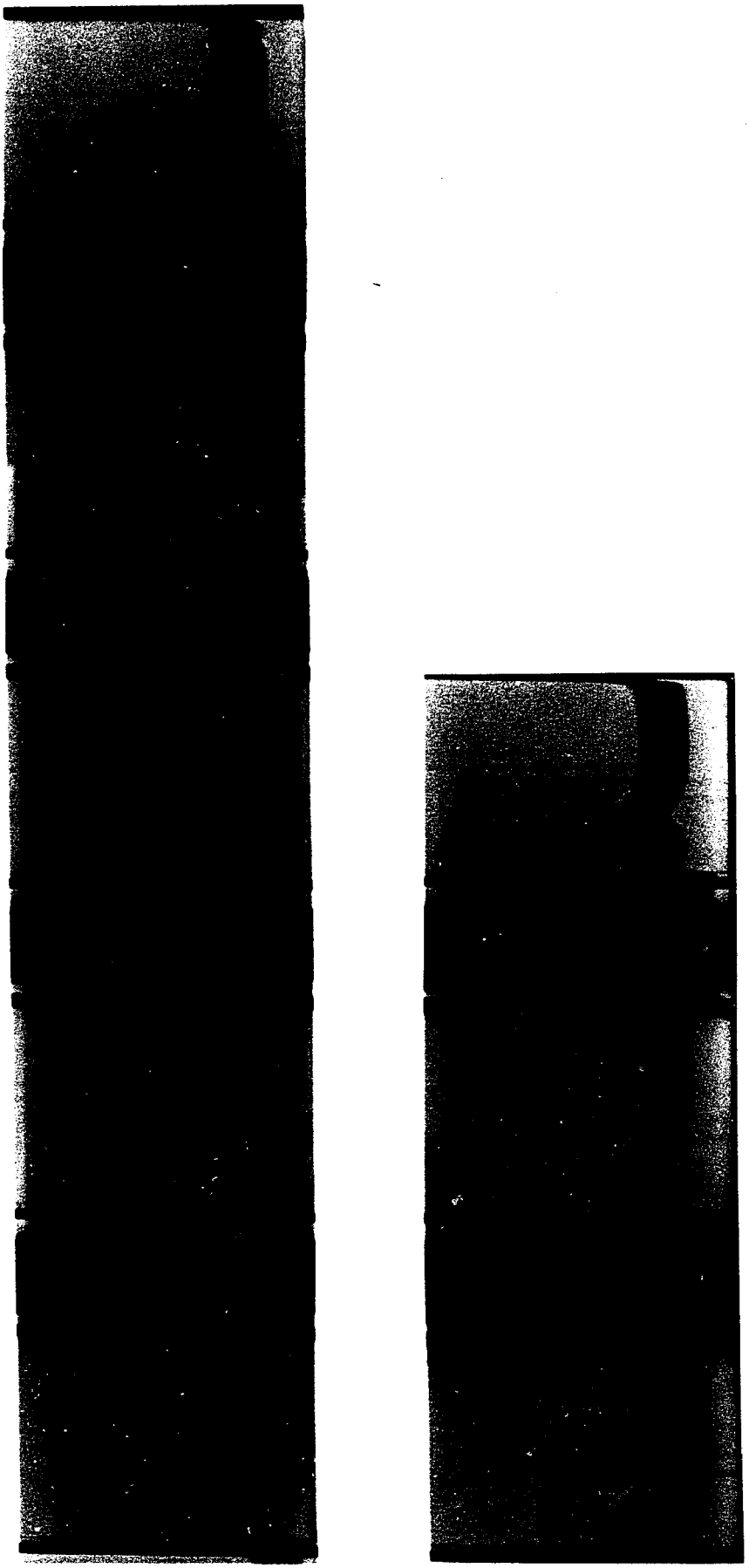


Figure 20. Ultracentrifuge analysis of ammonium sulfate serum fraction 5. 58,800 rpm, voltage 80, 3.5 sec. exposure at 8 minute intervals.

sedimenting at a much slower rate was determined to be in a ratio of approximately 4:1 in comparison with the faster moving component. Figure 21 shows an enlarged drawing of the third exposure of the series presented in Figure 20. Plainimeter analysis of the areas under the two peaks indicated the slower moving substance to be approximately four times more concentrated than the faster moving substance. It appeared that the component present in the PPLO serum fraction sedimented at about the same rate and was present in approximately the same concentration as the slower moving component in ammonium sulfate fraction 5.

c. Electrophoretic Analysis. Figure 22 shows the results of the electrophoretic analysis using ammonium sulfate fraction 5. The lower half of this figure shows the acetate strip stained with Ponceau S. Two bands of protein are readily apparent, one having an appreciably greater mobility than the other. Cutting the strip into the two segments and eluting the stain in N/10 NaOH followed by the addition of 0.1 ml of 40 per cent acetic acid per ml of eluate allowed quantitative comparison by spectrophotometry. The faster moving component was found to be in a concentration approximately 4 X greater than the slower moving component.

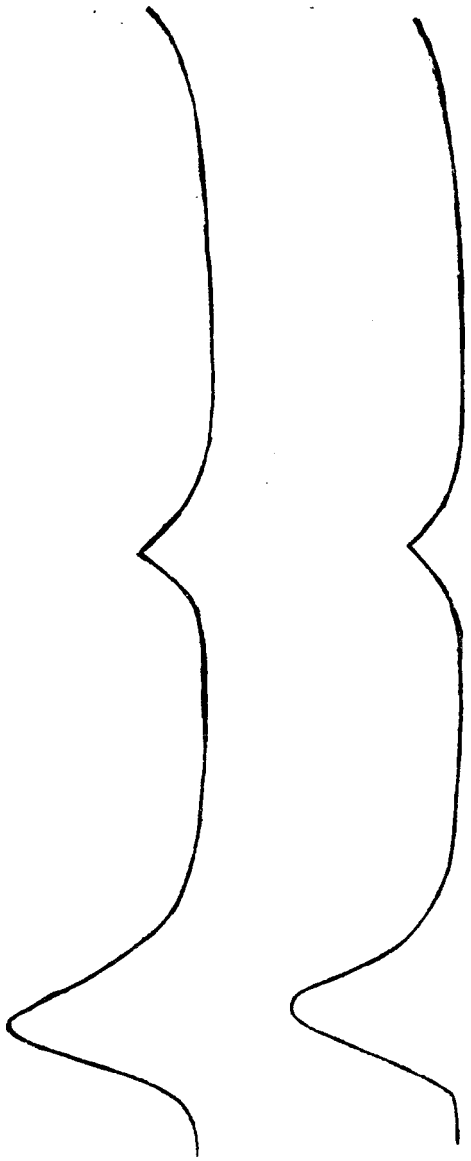


Figure 21. Enlargement of 3rd exposure seen in Figure 20, ultracentrifugation pattern of ammonium sulfate fraction 5.

Figure 22. Cellulose acetate electrophoresis of ammonium sulfate fraction 5. Upper half shows glycoprotein staining by the P.A.S. method. Lower half stained with Ponceau S, not cleared. The inverted V indicates the point of application of the serum fraction.



Figure 22. Upper half

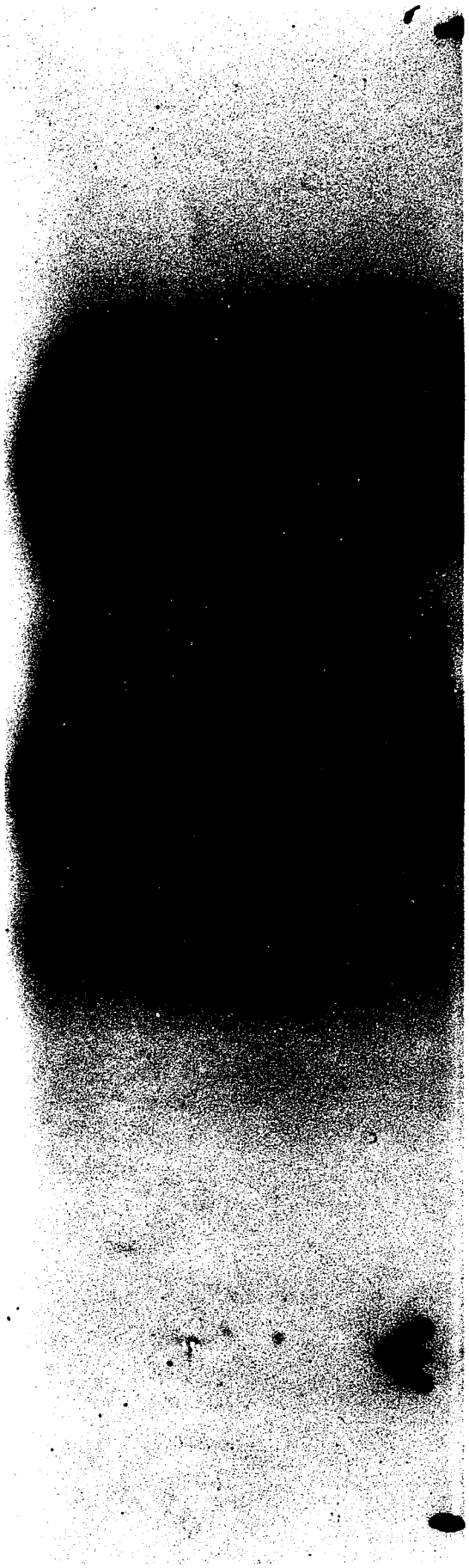


Figure 22. Lower half

The upper half of Figure 22 shows a duplicate trial which was stained with periodic acid-Schiff (P.A.S.) stain which identifies glycoprotein. Only one component is seen to be evident in this preparation, this corresponding to the slower moving component which was present in lower concentration than the faster moving component.

On the basis of relative concentrations, the faster moving component found on electrophoresis is the slower sedimenting component demonstrated by ultracentrifugation. This component has no carbohydrate associated with it since it failed to take the P.A.S. stain. The faster sedimenting component and the slower migrating component appear to be the same on the basis of comparative concentration. This would appear to be glycoprotein since it showed a positive reaction to the P.A.S. stain.

DISCUSSION

Five basic substances are necessary for the multiplication and survival of microorganisms as they are commonly propagated *in vitro* in artificial culture media. These are: (1) a source of nitrogen, (2) a source of carbon, (3) water, (4) salt, and (5) accessory growth factors. Of these the last in most instances are not well categorized.

Trichomonads which have been studied most intensively *in vitro* are those which in their natural environment are in a sodium chloride concentration of approximately 0.85 per cent (0.15 M). Witte (1933) included 0.3 per cent NaCl in the original medium in which he first grew *T. foetus* bacteria-free. Salt has been a constituent of culture media which have been routinely used for the cultivation of these flagellates. Wenrich (1945, 1946) stipulated the use of a salt solution, either full or half strength Ringer's, for the cultivation of intestinal trichomonads from man, certain rodents, and frogs. It was somewhat surprising to learn that salt had not been included in the STS medium of Kupferberg *et al.* (1948). Simple experiments described here demonstrated a significant increased growth response of *T. foetus* strain M grown in

the basal medium (STS) containing 0.5 per cent (0.09M) NaCl as compared with the response in the same medium without salt.

Suzuoki and Suzuoki (1951), while studying the carbohydrate metabolism of T. foetus, found the oxygen uptake using glucose as the substrate to be maximal at a NaCl concentration of 0.19 to 0.26 M. Ninomiya and Suzuoki (1952) also demonstrated the optimum conditions for respiration in the presence of glucose to include 0.1 to 0.2 M concentration of salt. It might thus be reasoned that NaCl is involved in some way with carbohydrate metabolism as evidenced by the increased oxidation of glucose when it is present in the environment. This would explain the faster growth rate demonstrated in the medium containing 0.5 per cent NaCl.

Cysteine was demonstrated to have no influence on the cell population of T. foetus strains M, L and UT when 5 per cent horse serum was employed in the basal medium and incubation was at a reduced oxygen tension. This was not true for T. vaginalis as average counts of 3120 and 1029 cells per mm^3 were observed in media with and without cysteine respectively. This probably reflects a basic difference in oxidation pattern of these two organisms.

Trichomonas foetus being a facultative anaerobe can metabolize over a fairly wide range of oxygen tension while T. vaginalis is more nearly anaerobic in its requirements and the cysteine serves as a reducing agent. The CPLM medium of Johnson and Trussell (1943), which was the first reported to contain cysteine, was originally developed for growing T. vaginalis. Since it was found to support T. foetus also, with proper modification of pH, cysteine has been included in subsequent formulations. Unless it is present and available in trypticase or as a constituent of serum it can be considered not to be required for survival of either T. foetus or T. vaginalis. Although its effect on the growth rate of the latter is highly stimulatory its action may be entirely in the category of a reducing agent.

Sanders (1957) was the first to report the ability of cholesterol and Tween 80 to replace whole serum in a comparatively well defined medium. His medium contained a vitamin mixture and an amino acid mixture studied by Cowperthwaite et al. (1953) in the nutrition of Herpetomonas culicidarum, in addition to trypticase and ribonucleic acid. Cailleau's (1938a) claim that cholesterol was an essential growth factor for T. foetus was

not conclusively demonstrated by her and the report of Sprince and Kupferberg (1947b) indicated that cholesterol was not the active material in their ether solubles required for the cultivation of T. vaginalis. In the current work the inability of cholesterol alone or in combination with Tween 80 to support growth of T. foetus in the modified basal medium described would indicate the need for other substances which are supplied by serum or some serum fractions. Assuming the extraction procedure of refluxing lyophilized serum for 24 hours with acetone, benzene, chloroform or ether to be adequate and efficient for the extraction of cholesterol and other lipids, while at the same time effecting no protein denaturation, it would appear that lipid substances present in serum are not necessary for the growth of T. foetus. The ability of lyophilized serum so extracted to support T. foetus in numbers statistically no different than unextracted lyophilized serum would tend to indicate a lack of need for lipids. The absence of a positive test for sterol by either the Lieberman or the Salkowski reaction in any such sample so extracted would lend further support to the belief that cholesterol is not essential in the nutrition of this organism.

In addition to these findings, the lack of a positive test for sterol in the solubles left after heating to 100 C for 20 minutes and in all fractions of serum obtained by dialysis would, since these materials all supported growth of T. foetus, indicate no need for cholesterol in the metabolic pattern. Clausen (1953) working with an aqueous extract of inspissated serum concluded the necessary factor in serum was a glycoprotein and not cholesterol or a lipoprotein.

Cholesterol is known to have the ability to detoxify a medium containing fatty acids. A. Lwoff (1947) demonstrated that Moraxella lacunata could not grow in ordinary nutrient broth. However, if serum or cholesterol were added multiplication occurred. In this case it was concluded that cholesterol did not act as a growth factor but rather acted by neutralizing the fatty acids in the broth. Snyder and Meleney (1942) first demonstrated the need for cholesterol in the cultivation of E. histolytica and this indispensability was confirmed by Griffin and McCarten (1949). However, the latter questioned whether the need was as a true metabolic requirement or if the cholesterol was serving as a detoxifying material.

In the current work ascorbic acid added to the culture medium produced varying results depending on the serum fraction used in place of whole serum. It was without effect in those instances where intact whole serum was used or where medium containing whole serum was autoclaved after addition of the serum. In no instance was it shown to be indispensable for the growth of the trichomonads used. When used in 0.1 per cent concentration with ammonium sulfate fractions 4, 5 and 6 a marked growth response was observed, a comparable response being absent in medium containing fractions 1 or 3. The response observed with fraction 5 was the most marked although the difference in trichomonad count between the basal medium containing 5 per cent of fraction 4, 5 or 6 with and without 0.1 per cent ascorbic acid was significant at the 1 per cent level. This concentration of vitamin C with 10 per cent of fraction 5 in the basal medium supported growth of all three strains of T. foetus to a level that was no different statistically from that of the basal medium with 5 per cent intact serum. Thus, using fraction 5 in 10 per cent concentration and supplementing it with ascorbic acid, a medium was obtained which equaled that of the basal medium containing whole

serum. There was a difference among strains but this was shown to be a strain difference and not a medium difference since the same difference existed regardless of whether the medium contained 5 per cent intact serum or 10 per cent fraction 5 and 0.1 per cent ascorbic acid. This probably reflects to some degree an adaptation to artificial culture medium in general and to the basic STS medium in particular. Strain M had been propagated in the laboratory on STS medium for some time and thus had an appreciable time to adapt. Strains L and UT were of much more recent vintage and had been carried on CPLM medium when cultivated but for the most part had been maintained frozen.

Ascorbic acid was shown to have no affect on trichomonad counts when added to intact horse serum or when added to the basal medium which had been autoclaved containing 5 per cent horse serum. All counts for six subcultures in medium containing serum with and without ascorbic acid averaged 2802 and 2400 cells per mm^3 respectively. These did not differ significantly. In medium containing autoclaved serum with and without ascorbic acid the averages were 1479 and 1517 cells per mm^3 respectively. These likewise were not significantly

different but there was a highly significant difference between the media containing heated and unheated serum. Autoclaving was observed, as was heating serum to 100 C for 20 minutes, to remove, to make unavailable or to make less available something in serum which is not essential for the maintenance of T. foetus but which does contribute to its rate of reproduction. Ascorbic acid in 0.1 per cent concentration could not replace this substance.

These findings are in essential agreement with those of Cailleau (1938a, 1938b, 1939c), Johnson (1947) and Rieck (1953, 1956) in that under certain conditions ascorbic acid was found to have a stimulatory effect on growth rate but was not indispensable for continued subculture. Rieck (1953, 1956) particularly recommended the use of supplemental vitamin C for the cultivation of T. foetus where horse serum was used in the culture medium. In the present study while the counts were higher in the tubes containing intact serum and ascorbic acid they were not significantly higher.

Pantothenic acid was observed to have no influence on the growth response of T. foetus strain M when used alone

or in combination with ascorbic acid in media containing ammonium sulfate fractions 1, 3, 4, 5, or 6. A need for added pantothenate had been demonstrated only for T. vaginalis by Kupferberg et al. (1948) but this only when serum concentration was reduced to 0.5 per cent.

Horse serum could be separated into 6 fractions by the ammonium sulfate fractionation method of Cohn et al. (1940a), five (1, 3, 4, 5, and 6) of which alone were able to support the multiplication of T. foetus in sustained serial subculture. With fractions 1, 3 and 6 growth rate increased as the concentration of the fraction increased. In this regard the pattern of the growth response simulated that seen with increasing concentrations of intact serum. This was not the case with fractions 4 and 5 (see figure 11). With these no increase in growth response was observed with concentrations greater than 10 per cent. At concentrations of 5 per cent and after 48 hours incubation only fraction 5 showed an increased count with time. Each of the other fractions gave counts at 48 hours which were as high as or higher than at 120 hours (see figure 12). Fraction 5 demonstrated the greatest growth support when ascorbic acid was added in a concentration of 0.1 per cent (see figure 13).

This fraction used in 10 per cent concentration and with 0.1 per cent ascorbic acid was found to be able to replace intact serum in the basal medium.

Cohn et al. (1940a) indicated that the fraction of serum precipitated at an ammonium sulfate concentration of 2.8 moles/liter (68 per cent saturation) contained crystallin albumin, hemocuprein, choline esterase, glycoprotein and phosphate. This represented 14 per cent of the total protein of which 99 per cent was water soluble. Of the 6 fractions obtained from horse serum by ammonium sulfate fractionation in the current work, fraction 5 corresponded to that fraction of Cohn et al. (loc. cit.) precipitable between 0.62 and 0.68 saturation. On analysis for carbohydrate and protein this fraction was found positive and gave a positive sterol reaction by the Salkowski Test but was negative by the Lieberman Test. The significance of this difference in the sterol test is not known. Both electrophoresis and ultracentrifugation showed this fraction to contain two components, both of which were protein and one of which in addition appeared to be conjugated to carbohydrate, since on the electrophoretic sample it gave a positive P.A.S. reaction. This fraction supported multiplication of T.

foetus strain M in concentrations as low as 2 per cent, the lowest concentration tested. In a concentration of 10 per cent and with 0.1 per cent ascorbic acid it supported the multiplication of T. foetus strains M, L and UT in numbers that were not significantly different from those obtained when intact serum was used. In terms of total protein, assuming the fraction 5 obtained here to be somewhat comparable to that obtained between 0.62 and 0.68 ammonium sulfate saturation by Cohn et al. (1940a), using fraction 5 in 10 per cent concentration introduced only approximately one-fourth as much as the use of whole serum in 5 per cent concentration. No attempt was made to concentrate any of the fractions but it is possible that in view of the high water solubility reported for fraction 5 this could easily be accomplished. The fact that serum could be fractionated into a number of components each of which alone, and in a concentration no greater than would be available in whole serum, could support the sustained subculture of T. foetus indicates the mosaic nature of whole serum for the growth support of this organism. This finding indicates that a number of substances in serum all probably contribute to its effect when used intact. On the basis of lipid extractions of lyophilized serum using acetone, benzene, chloroform and ether and on the basis of a negative cholesterol test on fractions obtained

by dialysis and on solubles remaining after heating, it is felt that proteins supplied by serum are its most important contributions. It would appear that something which contributes to the multiplication rate of T. foetus is lost on dialysis of whole serum. Dialysate of 72 hour dialyzed serum added to the dialyzed portion did not restore the full growth promoting power of whole serum. It is possible that some minor irreversible change occurred in a protein constituent as a result of salt loss and this was responsible for lessened growth promoting ability.

Human serum albumin, fraction V prepared by the ethanol fractionation method and obtained from the Michigan Department of Health, was unable to sustain T. foetus. This is somewhat paradoxical since fraction 4 obtained by ammonium sulfate fractionation, according to Cohn et al. (1940a), consists largely of crystallin albumin and was able to sustain growth of T. foetus. Sprince and Kupferberg (1947b) indicated that serum albumin was one of the active components of their aqueous ether-insoluble phase. Failure to maintain growth with bovine albumin obtained from Armour and Company, alone or with the acetone, benzene, chloroform or ether-solubles collected from reflux extraction of lyophilized serum further clouds the picture.

Fraction 5, obtained by ammonium sulfate fractionation of horse serum, was shown by ultracentrifugation to consist of two components, one of which demonstrated sedimenting properties similar to the single component seen in PPLO serum fraction (Difco). Both fraction 5 and PPLO serum fraction were able to sustain growth of T. foetus and thus it would appear that the common component might be contributing to this multiplication. It was not expected that the PPLO serum fraction would show a single component since, according to Smith and Morton (1951), it represented everything precipitable between 0.62 and 1.00 saturation. Ammonium sulfate fraction 5 which represented only those materials precipitable from 0.62 and 0.68 saturation on the other hand presented two distinct components both by ultracentrifugation and by electrophoresis. Two things might explain this difference. First, no definite information could be obtained from Difco concerning the preparation of their PPLO serum fraction other than it represented a fraction obtained by ammonium sulfate precipitation according to the method of Smith and Morton (1951). Second, it was shown by the ring precipitation test that the PPLO serum fraction was of bovine origin and not of equine origin as was fraction 5. Thus the species difference may explain the unexpected difference in the number of components found on ultracentrifugation. Nevertheless since

both fractions supported multiplication of T. foetus and since both appeared to contain a common component it is surmised that this component contributed to the multiplication.

The current work is the first report on the ability of certain serum fractions, obtained by ammonium sulfate precipitation, to replace whole serum for the in vitro cultivation of T. foetus. Fraction 5 used in 10 per cent concentration and with 0.1 per cent ascorbic acid supported growth of three different strains of this flagellate in numbers equal to those found with whole serum. This affords material much less complex than whole serum on which to perform further purification and fractionation. Fraction 5 would be an ideal material on which to perform continuous flow paper electrophoresis. Fractions collected by this procedure could be tested for biological activity in terms of growth promotion and could be qualitatively analyzed and quantitatively assayed. Enzymatic and acid hydrolysis are also procedures which could be performed using fraction 5 as the starting substance. Further purification might be attempted using a nucleic acid or a protamine. All of these and other procedures should contribute to a better understanding of the nature of the active substances in fraction 5.

The inability of ammonium sulfate fraction 5 with 0.1 per cent ascorbic acid to support the growth of T. vaginalis

reflects a difference in nutritional requirements of this species. The observations that this species grew much more poorly in medium devoid of cysteine HCl than in the same medium containing this amino acid and that all three strains of T. foetus used grew equally well with and without cysteine HCl also indicate a difference in culture requirements between these species.

Until trypticase can be replaced in the base medium by chemically defined entities the exact contribution of serum will not be learned. From available data it appears that some protein or protein derivative and possibly some lipid or lipid complex are the basic essentials for maintenance but that probably a mosaic of factors contribute to the growth rate observed when intact serum is used in artificial culture media.

SUMMARY AND CONCLUSIONS

The primary objectives of this work were to learn if whole serum could be separated into fractions which alone could be used to replace intact serum as a source of accessory growth factors for the growth of T. foetus, and, if this were possible, to learn something of the nature of the components of such fractions. There has been some question concerning the role of cholesterol as a replacement for whole serum and it was hoped that additional information might be obtained on this problem. In addition certain substances were tested for their ability to replace whole serum in the growth medium and certain manipulative procedures were performed with serum to learn, from a quantitative standpoint, what effect these had on growth promoting ability.

The basal medium used throughout the study was the STS medium of Kupferberg et al. (1948) modified by the addition of 0.5 per cent sodium chloride. The addition of this amount of salt allowed T. foetus strain M to multiply at a significantly greater rate than in the same medium but without added salt.

Cholesterol, with or without Tween 80, was unable to replace serum in the basal medium. Likewise ascorbic acid

and pantothenic acid alone or in combination were unable to sustain growth of T. foetus in the absence of serum. Hemoglobin in various concentrations also was unable to support multiplication. The M, L and UT strains of T. foetus were unaffected by the deletion of cysteine HCl from the basal medium. This amino acid was shown to be stimulatory but not essential for the growth of T. vaginalis.

Treatment of horse serum with 95 per cent ethanol to effect complete protein precipitation removed the growth promoting properties from the serum.

Dialyzing horse serum vs. distilled water in the cold for 18 days resulted in water soluble and water insoluble components both of which individually supported growth of T. foetus strain M but at a slower rate than when combined. Growth rate using the combined fractions was significantly slower than when intact serum was used. Dialyzing horse serum for only three days yielded a slight precipitate which was readily solubilized by the addition of a few crystals of NaCl. Both the dialyzed serum and the dialysate supported growth of T. foetus but neither in quantity equal to that of intact serum. The presence of protein in the dialysate, as revealed by slight coagulation on heating, indicated faulty apparatus in the dialyzing procedure. The addition of

dialysate to the dialyzed serum did not restore growth promoting ability equal to that observed with whole serum.

Heating serum to 100 C for 20 minutes or adding serum to the basal medium in 5 per cent concentration and autoclaving for ten minutes reduced but did not completely remove growth promoting properties. The addition of ascorbic acid to the autoclaved medium containing serum was without effect on its growth supporting ability.

Reflux extraction of lyophilized horse serum with acetone, benzene, chloroform or ether removed none of the growth promoting qualities of such extracted sera. The extracted sera gave negative tests for cholesterol thus indicating that this substance is not essential for the growth of T. foetus, at least when other serum components are available. The solvent extracts, all of which except the benzene extract gave a positive test for cholesterol, emulsified in 5 per cent bovine albumin did not support multiplication.

Fractions II + III, IV-1 and IV-4 of human plasma prepared by the ethanol fractionation method of Cohn et al. (1940b, 1946) were able to support sustained growth of T. foetus strain M but at a much slower rate than with intact

serum. Of the three fractions, II + III supported the most growth. Solubility problems with these fractions resulted in the use of the ammonium sulfate fractionation method for additional fraction preparation.

Difco's PPLO serum fraction was capable of supporting growth of T. foetus strain M although the rate of growth was somewhat slower than with intact serum. Ultracentrifugation of this fraction revealed a single component which appeared to sediment at about the same rate as albumin.

Five of the six fractions derived by ammonium sulfate fractionation of horse serum were individually able to support growth of T. foetus strain M. Fraction 5, representing insolubles between 0.62 and 0.68 saturation, was the best of the five fractions. When used in 10 per cent concentration and with 0.1 per cent ascorbic acid it supported growth of T. foetus strains M, L and UT equal to that found when intact serum was used. Trichomonas vaginalis was not supported by this fraction alone or with ascorbic acid in the basal medium. This fraction gave positive tests for carbohydrate, sterol and protein. By paper electrophoresis and by ultracentrifugation it was shown to consist of two components whose ratio was

approximately 4:1. The slower component electrophoretically corresponded to the faster sedimenting component in the ultracentrifuge and was in lower concentration than the other component. It was shown to be glycoprotein by the P.A.S. method. The sedimentation pattern of the slower sedimenting component appeared almost identical with that of the single component seen in the PPLO serum fraction.

This gives for the first time a definite serum fraction which, with ascorbic acid, can be used to replace whole serum for the in vitro cultivation of T. foetus. This should allow additional purification and fractionation to determine more precisely the exact nature of the essential substances required for the growth of this flagellate.

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