THE EFFECT OF BACTERIA ON THE GROWTH OF PENTATRICHOMONAS HOMINIS

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ABSTRACT

THE EFFECT OF BACTERIA ON THE GROWTH OF *PENTATRICHOMONAS HOMINIS*

by Paul A. Tucker

The effect of bacteria on trichomonad populations both *in vivo* and *in vitro* has been observed by many investigators, but the nature of the effect has remained largely unexplored. The purpose of this research was to observe whether bacteria affect *Pentatrichomonas hominis* adversely through the production of toxins or whether they have a beneficial effect as a source of nutrients for the trichomonads.

Two strains of *P. hominis* were used; strain Huf-2 which had been in culture for six years and strain 5MS which had been recently isolated.

In order to observe whether bacteria exerted a toxic effect on the trichomonads, strain Huf-2 was cultured in modified Diamond's medium, a complete trichomonad medium, either with living bacteria or bacterial filtrates of *Escherichia coli*, *Streptococcus faecalis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Aerobacter aerogenes*, *Clostridium sporogenes*, and *Cl. perfringens*. Except where the bacteria over-grew the cultures in bacteria-trichomonad cultures, trichomonad populations were equal or nearly equal to those of the bacteria-free controls. When bacteria-free filtrates of bacterial cultures were added to trichomonad cultures, growth of trichomonads was equal or higher than that of the controls. There were no observable toxins produced by either the bacteria or their filtrates. Any inhibition noticed could be explained as competi-

tion for available nutrients or to overpopulation by the bacteria resulting in unfavorable physical conditions for the trichomonads.

In comparing trichomonad growth at different bacterial populations it became evident that a range of bacteria of about 10^9 per ml gave optimum trichomonad growth and that in future research of this nature some concern should be given the number of bacteria involved.

The ability of bacteria to supply trichomonads with various nutritional factors was first tested by raising both strains of *P. hominis* with *E. coli* in modified Diamond's medium from which the essential components (trypticase, yeast extract, glucose, and serum) had been singly deleted. *P. hominis*, strain 5MS, was able to utilize *E. coli* to replace trypticase, yeast extract, glucose, and serum; whereas, strain Huf-2 could only use the bacteria to replace glucose. It seemed that strain Huf-2 through extensive culturing had probably lost its major ability to utilize bacteria.

In order to better delineate the nutritional factors the bacteria were supplying to *P. hominis*, strain 5MS, a glucose-balanced salt solution enriched with a metals mix was used as the basic medium. This medium did not support the growth of trichomonads. To this deficient medium was added varying combinations of trypticase, alkali-hydrolyzed yeast RNA, vitamin mix, cholesterol, and TEM 4T (a source of long chained fatty acids) to test the minimum essential components in the presence of *E. coli*.

The trichomonads were found to be able to utilize *E. coli* as a source of vitamins, fatty acids, and RNA. Trypticase and serum had a common unknown growth factor or factors which did not permit the

simultaneous deletion of both from the medium. *E. coli* also failed to supply the cholesterol required by the trichomonads.

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PAUL A. TUCKER

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INTRODUCTION

The host-parasite relationship is an interesting biological phenomenon which is receiving the increased attention of parasitologists and others in allied biological fields, according to Cole (1955) and Stauber (1959). One aspect of this relationship - the interactions of symbionts within the host - was the general basis of this present study.

The purpose of this investigation was to determine: (1) whether bacteria plus their secretions can be utilized by trichomonads for food; (2) whether certain bacteria or their secretions inhibit trichomonad growth; (3) and whether either of the above effects are influenced by different bacterial population levels.

Amongst the many families of intestinal flagellates the choice of an organism for study was directed toward the Trichomonadidae, the family best known with respect to their nutrition and physiology. Within the family, Pentatrichomonas (=Trichomonas) hominis was chosen as the best known representative of mammalian intestinal inhabitants and one which could also be easily isolated and grown in axenic culture.

This study was undertaken in the hope that more information on the bacteria-trichomonads interaction in the host would lead to a better understanding of the trichomonad-host relationship.

Davaine (1860) described and named the first flagellate found in the intestine of man as Cercomonas hominis vars. A and B. For a number of years this name, as well as other names, was used to describe this organism until Kofoid (1920), after reviewing the literature, proposed that Cercomonas hominis var. B be given the name Trichomonas hominis (Davaine, 1860). After a detailed study of the structure of this trichomonad Kirby (1945) suggested that due to its constant five flagellar pattern it should be raised to a new genus denominated as Pentatrichomonas (Mesnil, 1914).

With the advent of axenic culturing of trichomonads, many investigators turned their attention to the problems of trichomonad nutrition and physiology. Cailleau (1936a, 1938) using a peptone-serum bouillon medium demonstrated the necessity of adding cholesterol and ascorbic acid for the growth of Trichomonas foetus and Trichomonas columbae. In determining whether cholesterol was a specific requirement, Cailleau (1936b, 1937) used 66 different sterols and sterol derivatives as additives to the medium and found that 18 of them supported growth of T. columbae. In 1939 Cailleau demonstrated that egg albumin, dead Shigella sp., and even unidentified bacterial contaminants could not support T. bactrachorum in the absence of cholesterol.

Johnson (1947) found that CPLM medium, modified to contain only 0.375% serum and 50% of the normal liver infusion complement, supplements of ascorbic acid, glutamic acid, and choline stimulated the growth of Trichomonas vaginalis.

Sprince and Kupferberg (1947a) devised a complex medium containing serum, trypticase and a series of possible chemically defined growth

factors. In trying to simplify their basic medium they found that trypticase (BBL) gave better and more consistent growth with T. vaginalis than Difco peptone. Trypticase, a pancreatic digest of casein, contained no detectable carbohydrates and appeared to be a purer source of nitrogenous products than most peptones.

Sprince and Kupferberg (1947b) later separated human blood serum into two fractions, an ether-soluble fraction and an ether-insoluble fraction. They demonstrated that both fractions were necessary for the growth of T. vaginalis in the above medium minus serum. Linoleic acid and an unknown heat-stable dialyzable factor were the essential nutrients in the ether-soluble fraction. Serum albumin was demonstrated to be one of the active nutrients in the ether-insoluble fraction. It is interesting to note that cholesterol was not defined as an essential nutrient of the ether-soluble fraction.

Kupferberg, Johnson, and Sprince (1948) felt that serum and trypticase had the effect of masking the importance of some of the added known growth factors in the highly complex medium of Sprince and Kupferberg (1947a). It was found that the serum concentration could be reduced from 5% to 0.5% if calcium pantothenate was added to the medium. Trypticase could be reduced from 2% to 0.25% if 5% serum was present. If 0.24% trypticase, 0.5% serum, and calcium pantothenate were used together no growth was noticed unless a 0.027% phosphate buffer was added. Thus for T. vaginalis they reported calcium pantothenate and phosphate to be essential growth factors in limiting amounts of trypticase and serum and that some unknown factors were common to both serum and trypticase.

Fourteen amino acids were found by Weiss and Ball (1947) to be essential for T. foetus in a serum-free medium. They also found that T. foetus responds well to proteins which have been partially digested. As proteins neared the amino acid stage their effectiveness rapidly decreased. This was interpreted by Weiss and Ball as a possible indication that T. foetus utilizes strepogenin or at least unknown peptides as a growth factor.

Sprince, Goldberg, Kucker, and Lowy (1953) reported the effects of ribonucleic acid and its nitrogenous constituents on the growth of T. vaginalis in the complex medium of Sprince and Kupferberg (1947a). They found that RNA could partially replace trypticase up to an optimal concentration of 0.1% RNA, but a higher concentration was inhibitory. Acid hydrolysis of RNA destroyed its growth-promoting activity; but alkaline hydrolysis had no affect on the activity. DNA under no condition was capable of promoting growth. A mixture of adenylic, guanylic, and uridylic acid substituted for 0.1% RNA supported good growth of the trichomonads. Nucleosides could not be demonstrated to appreciably sustain growth; and in media low in peptides and/or amino acids, ribonucleosides were inhibitory. The purine and pyrimidine bases of the above nucleic acids supported as much growth as the ribonucleotides. The authors hypothesized that the essential ribonucleotides could be formed by T. vaginalis from free purine and pyrimidine bases and the inactivity of the nucleosides might be due to the inability of the organism to phosphorylate nucleosides.

Sanders (1957), in the culture of T. foetus, confirmed the importance of cholesterol (Cailleau, 1938) by replacing serum with cholesterol and

Tween 80 in a medium where the primary ingredients were small quantities of trypticase, ribonucleic acid, a metal mix, vitamins, and amino acids.

Shorb and Lund (1959) in using a complex medium for the cultivation of T. gallinae demonstrated the presence of both an unknown trypticase and an unknown ribonucleic acid factor. No growth was seen when RNA was replaced with 40 different purines, pyrimidines, nucleotides and nucleosides. The active RNA factor was found to be in the contaminating protein of the RNA hydrolized yeast. It was found to be nondialyzable and amino acids alone or in mixtures could not replace this factor. The nondialyzable trypticase factor was analyzed using two-dimensional chromatography and was found to possibly be a large peptide but different from the RNA factor. They replaced serum by using cholesterol and a mixture of unsaturated and saturated fatty acids.

Lee and Pierce (1960) working with Hypotrichomonas acosta replaced serum with cholesterol and TEM 4T as a source of fatty acids. Alkali-hydrolized yeast RNA was replaced by a RNA-nucleotide mix. The trypticase was reduced as low as 0.25% in a medium whose major nutritional components were glucose, a metal mix, a nucleotide mix, cholesterol, TEM 4T, a vitamin mix, and a complex amino acid mixture.

Trichomonads have been shown to use a wide range of carbohydrates and the variation encountered among species was partially prevalent even among strains of the same species (Read, 1957; Lee and Pierce, 1960; Twohy, 1959).

As far as nutritional studies have progressed in the last twenty years, there are still many problems yet unsolved and much work remains

to be done on the interactions of many of the various nutritional factors. As von Brand (1952) concluded, "Obviously a multitude of possibilities exists in such a complex situation and, in most instances, it is at present impossible to decide definitely whether a certain effect was due to a direct or indirect action of a given nutritional component of the diet."

Pentatrichomonas hominis infects a wide range of animals. The natural hosts are the dog, cat, rat, mouse, golden hampster, man and thirteen other primates. Guinea pigs, ground squirrels, and chickens have served as experimental hosts (Levine, 1961).

Hegner and others studied the influence of diet upon populations of trichomonads in the intestine. It was observed that a diet heavy in protein caused trichomonad populations to diminish markedly and that high carbohydrate diets with a minimum of protein produced a fluorishing population of trichomonads (Hegner, 1923, 1924, 1927, 1933; Hegner and Eskridge, 1935; Wantland, 1954). Hegner and Eskridge (1937) noted the similar reactions of amoebae in rats to high carbohydrate and protein diets. They attributed the change in trichomonad populations to the altered composition of the bacterial flora resulting from change of the diets. Proteolytic anaerobic bacteria seemed to be associated with the decrease in the trichomonad populations and acidophilic bacteria favored the increase of trichomonads (Ratcliffe, 1928; Hegner, 1932, 1937).

Although trichomonads were isolated and grown in vitro with bacteria in the early thirties, the study of the specific effects of different bacteria on the in vitro growth of trichomonads was not really begun

until the 1940's. Johansson, et al. (1947) studied the effect of various bacteria upon the growth of Trichomonas foetus. They employed a modification of Schneider's (1942) medium consisting of an egg slant and overlay. Serum, glucose, and whole egg were the primary sources of nutrients which supported adequate growth in the absence of bacteria. There was no attempt to control or determine bacterial populations. The bacteria found to be inhibitory were Corynebacterium diphtheriae, C. pyogenes, C. xerosis, Staphylococcus aureus, Staph. albus, Streptococcus bovis (five strains), Strep. faecalis (two strains), Strep. salivarius, Strep. hemolyticus, Shigella dysentariae, Shig. paradysenteriae, Escherichia coli, and Aerobacter aerogenes. Bacteria that had no appreciable effect were Vibrio comma, Neisseria catarrhalis, Strep. pyogenes, C. hofmannii, C. renale, Bacillus subtilis, Pseudomonas aeruginosa, Sarcina sp., and nine unknown cultures of diphtheroids. C. equi alone prolonged the survival of the trichomonads beyond that of the bacteria-free controls. Johansson, et al. (1947) also used bacterial filtrates and heat-killed organisms in in vitro cultures of trichomonads which produced similar effects on the growth of the trichomonads to those noticed with the living bacteria. He explained the results which ranged from accelerated growth to inhibition as varied: (1) hydrogen ion concentrations of the culture, (2) production of toxins and (3) recruitment of additional trichomonad nutrients by bacterial enzymatic action on the medium.

Hitchcock (1948) demonstrated an inhibiting effect of trichomonad secretions upon bacteria. Trichomonas foetus, strains BR, O, and C, inhibited Salmonella pullorum. Strain BR inhibited Corynebacterium

renale and Salmonella schottmuelleri. T. vaginalis had no effect on any of ten species of bacteria tested.

Pray (1952) used the medium of Sprince and Kupferberg (1947a) consisting of serum, trypticase, maltose, and cysteine, plus vitamin, purine and pyrimidine supplements to study T. vaginalis. This medium supported excellent growth in the bacteria-free T. vaginalis controls. Bacterial populations were not controlled or counted. The bacteria which curtailed multiplication of the flagellate and the life of the cultures were Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa, Salmonella schottmuelleri, S. paratyphi, and Proteus mirabilis. Brucella suis, Streptococcus lactis, Ps. fluorescens, Alcaligenes faecalis, Sarcina lutea, and Bacillus subtilis caused a moderate inhibition of trichomonad growth. Staphylococcus aureus and Staph. albus prolonged the life of the cultures although they did not increase the total number of the trichomonads over that of the control. Bacterial filtrates produced no detectible inhibition on the trichomonads. Recently isolated trichomonads and the older cultural forms were the same in their reactions to the various bacteria. Pray concluded that the limiting factor in the trichomonad growth with bacteria was the low amount of available carbohydrate as a result of the competition with bacteria for the carbohydrate present in the medium. In the presence of both abundant sugar and bacteria trichomonad populations may be higher than in the bacteria-free controls.

GENERAL MATERIALS AND METHODS

Two strains of Pentatrichomonas hominis were used in this study. P. hominis, strain Huf-2,[1] was used through the early part of this study and P. hominis, strain 5MS,[2] was used in the latter part of the research. The bacteria[3] used were Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Aerobacter aerogenes, Streptococcus faecalis, Clostridium sporogenes, and Cl. perfringens. All of the bacteria were without strain designation or known history.

Routine cultures of trichomonads were maintained in a modification of Diamond's (1957) medium consisting of 2% trypticase, 1% yeast extract, 0.5% maltose or glucose, 0.1% L-cysteine hydrochloride, 0.1% agar and 5% inactivated calf serum maintained at about pH 6.6 in 0.011M dibasic sodium phosphate. This medium produced optimum growth with both strains of P. hominis.

The minimum medium for the maintenance of E. coli was a glucose-salts synthetic medium (Pelczar and Reid, 1958) consisting of 0.5% glucose, 0.5% sodium chloride, 0.02% magnesium sulfate, 0.1% ammonium acid phosphate, 0.1% dibasic potassium phosphate, 0.1% agar and brom cresol purple as a pH indicator. This medium would not support the growth of P. hominis.

When effects of pH on the trichomonad-bacteria association were investigated, all values were determined with a Beckman zeromatic pH meter. Later when it was found that variations of less than one pH

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[1] Isolated by Dr. Louis Diamond from human feces on March 26, 1956.

[2] Isolated from dog feces on October 4, 1960, at Michigan State University.

[3] Kindly supplied by Miss Lisa Neu, Department of Microbiology and Public Health, Michigan State University.

unit were not affecting the results, brom cresol purple indicator was used in the medium to follow the changes of pH.

Trichomonads to be used for inoculation were cultured for 36-48 hours in a 25 x 150 mm screwcap test tube containing 20 or 40 ml of modified Diamond's medium. The cultures were centrifuged and the supernatent discarded. The organisms were either re-suspended in a basic portion of the experimental medium or were washed with a Kreb's-Ringer's phosphate solution and recentrifuged before adding the experimental medium. In all experiments the inoculum of trichomonads was adjusted to give a final concentration in growth tubes of approximately 1×10^5 organisms per ml. The inoculum of bacteria was 0.1 ml of a twenty-four culture per 5 ml of medium. All organisms were cultured at 37°C and the time of culturing varied with the particular experiment.

The final volume, including the inoculum, was 5 ml of experimental medium in each growth tube except as otherwise noted. In most experiments four or five growth tubes were used to test each nutritional component in order that experimental variation could be evaluated. The organisms were killed and preserved for counting by using a final concentration of approximately 1% formalin in each tube.

The formalin-killed trichomonads were mixed thoroughly, and samples for counting were withdrawn in a Pasteur capillary pipette and plated under a Levy hemacytometer with an improved double Neubauer ruling. The population levels of the bacteria were determined by serial dilutions of a non-formalized culture. Each of the dilutions were inoculated into nutrient agar using a standard pour plate technique.

Various concentrations of a penicillin-streptomycin antibiotic solution were used in each experiment in order to hold the bacteria at favorable population levels. Without the antibiotics it was found that the bacteria outgrew the trichomonads in the experimental tubes to the detriment of the trichomonads. Effective concentrations had to vary with different media.

Routine inoculations were made of bacteria-free trichomonad cultures into brain-heart infusion broth in order to test for bacterial contamination. All possible care was taken against contamination with foreign organisms or materials.

The F-test was used in all tests of significance and the standard error of the mean is given with each average growth value.

EXPERIMENTS AND OBSERVATIONS

I. The Effect of Bacteria on the Growth of *Pentatrichomonas hominis*, Strain Huf-2, in Modified Diamond's Medium.

Since it has been reported that certain species of bacteria influence the growth of trichomonads in complete media (Johansson, *et al.*, 1947; and Pray, 1952), an attempt was made to test the effects of five enteric bacteria upon the growth of *P. hominis*.

Materials and Methods. Due to the great number of experimental culture tubes necessary if all five bacteria were tested at one time, Experiment I was divided into two series which were run at different times. In series 1 *Escherichia coli*, *Streptococcus faecalis*, and *Proteus vulgaris* were used as test bacteria, and in series 2 *Pseudomonas aeruginosa* and *Clostridium sporogenes*.

Sterile modified Diamond's medium was prepared in 100 ml quantities in each of 4 flasks for series 1 and in each of 3 flasks for series 2. Each of the test bacteria was inoculated into a separate flask and an equal amount of sterile water was added to one flask of each series to serve as a bacteria-free control. Six hours later for series 1 and 8 hours later for series 2, a penicillin-streptomycin solution was added to each flask to give a final concentration of 6000 units of each antibiotic per ml. A series of 4 ml tubes were prepared from each flask. A 1.0 ml inoculum of trichomonads suspended in fresh Diamond's medium was added to each tube. At given intervals of time, designated by the points in Fig. 1, a pH reading was taken and the remaining trichomonads were killed and counted. The value for each point represents the average count of 3 tubes.

Results. As seen in Fig. 1 maximum growth of the trichomonads

with four of the bacterial species was attained at about 40 hours. The exception was S. faecalis where maximum growth occurred in 24 hours. Only the trichomonads grown with Ps. aeruginosa had populations equal to that of the bacteria-free controls, but trichomonads still achieved relatively heavy populations with all other bacteria except S. faecalis. The F-test comparing maximum trichomonad populations showed a significant difference between the trichomonad population grown with all bacteria except Ps. aeruginosa and that of the controls at the 1% level of probability.

The pH of the E. coli-, P. vulgaris-, Ps. aeruginosa-, and Cl. sporogenes-trichomonad cultures was similar or higher than that of the controls over the range of the experiment (Fig. 1). The very low growth of trichomonads with Strep. faecalis probably resulted from bacterial overgrowth which rapidly depleted the nutrients and lowered the pH prematurely for optimum trichomonad growth.

Discussion and Conclusions. The results of culturing various bacteria with P. hominis in complete medium has confirmed the effects noticed by Johansson, et al. (1947) with Trichomonas foetus and Pray (1952) with Trichomonas vaginalis in which moderate to heavy inhibition of trichomonad growth was observed with most of the test bacteria. Although the magnitude of the effects was different, this is probably due to the differences in trichomonad species, the medium used by the different authors, and the concentration of bacteria present. In this experiment the reduced populations of the trichomonads grown with E. coli, P. vulgaris, and Cl. sporogenes most probably resulted from bacterial-trichomonad competition for the available nutrients rather

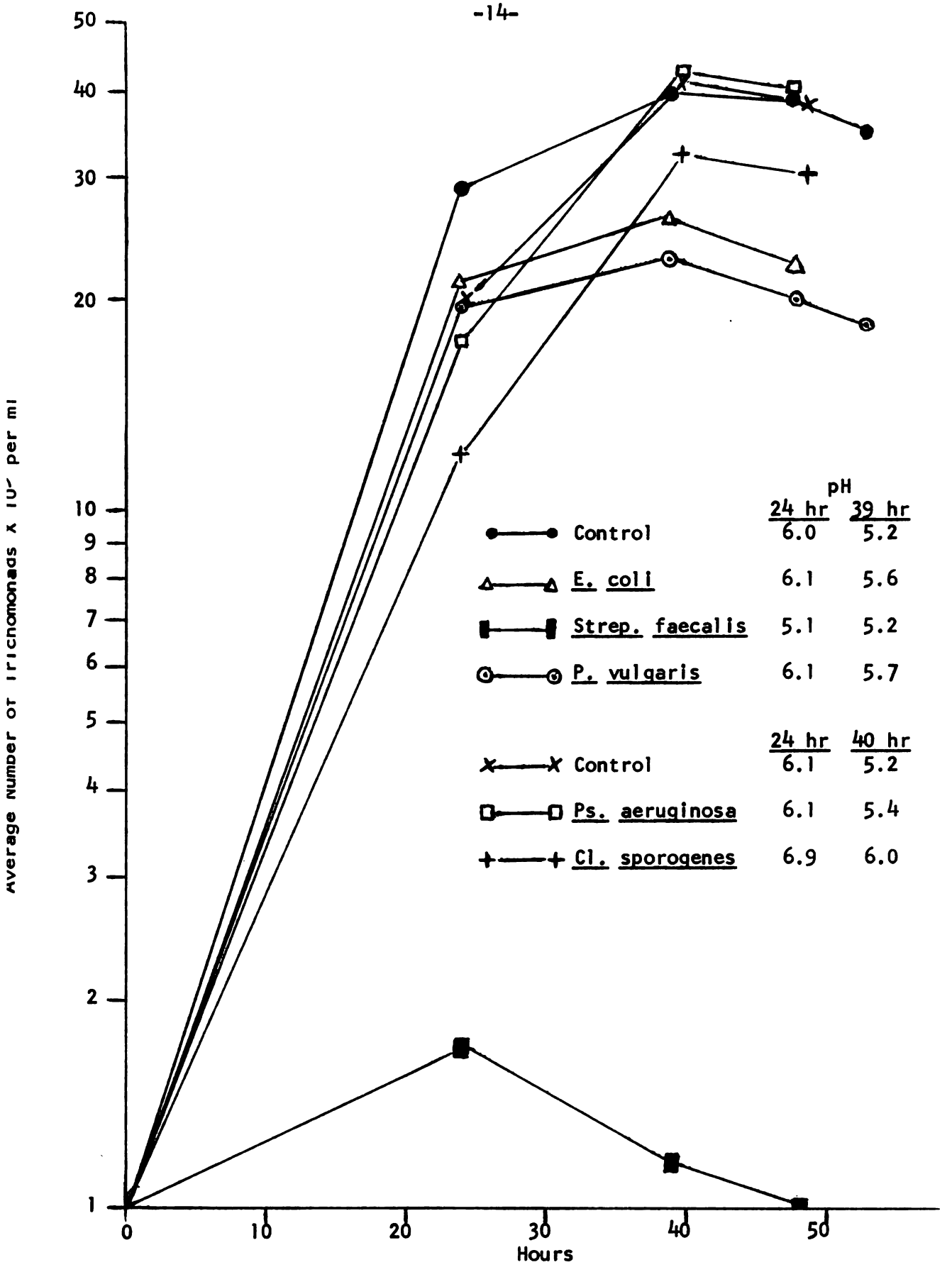

Fig. 1. Growth of P. hominis, Strain Huf-2, in Diamond's Medium with Various Bacterial Species.

than the production of a bacterial toxic factor.

II. The Effect of Bacterial Filtrates on the Growth of P. hominis, Strain Huf-2, in Modified Diamond's Medium.

It was felt that filtrates from a high concentration of bacteria would show the presence of any toxic factors by inhibiting the growth of P. hominis in a medium suitable for the growth of trichomonads.

Materials and Methods. The bacteria from which the filtrates were obtained were E. coli, Cl. perfringens, P. vulgaris, and Aerobacter aerogenes. The filtrates were obtained from bacteria cultured in 20 or 40 ml lots of modified Diamond's medium for 48 hours. The cultures were adjusted to pH 7.0 with 1 N NaOH and centrifuged to remove some of the bacteria and other large particles. The supernatant fluid was filtered through a Seitz bacteriological filter to remove the remaining bacteria. The filtrates were added to tubes of modified Diamond's medium in sufficient quantity to make a 10% concentration by volume before the trichomonads were inoculated into the medium and cultured for 40 hours. Controls were grown in equal volumes of modified Diamond's medium.

Results. The results in Table I indicate that the filtrates of E. coli and Cl. perfringens enhanced growth above that attained by the filtrate-free controls and that none of the filtrates inhibited growth.

Discussion and Conclusions. The use of bacterial filtrates showed no evidence of toxins or toxic materials being secreted by bacteria. Bacteria-free preparations from bacterial cultures either had no effect or slightly stimulated the growth of P. hominis. The slight increase in growth from added E. coli and Cl. perfringens filtrates (Table I)

TABLE I. Growth of *P. hominis*, Strain Huf-2, in Modified Diamond's Medium Containing 10% Bacterial Filtrates

Exp.	Bacteria	No. of tubes	With Filtrates ± S.E.[a]	No. of tubes	Without Filtrate ± S.E.[a]	F Value
1	*E. coli*	4	47.05 ± .47	4	41.43 ± .55	59.32*
1	*Cl. perfringens*	4	48.94 ± .55	4	41.43 ± .55	92.76*
2	*P. vulgaris*	4	46.84 ± .32	4	45.40 ± .53	3.42
2	*A. aerogenes*	4	46.02 ± .26	4	45.40 ± .53	1.08

[a] Average number of trichomonads X 10^5 per ml.

* Significance at the 1% level of probability.

could be due to the presence of a bacterial metabolite which enhances growth. Previous observations on the P. hominis-bacterial association (Fig. 1) showed no evidence of the bacteria producing strong toxins or even a marked inhibition of trichomonads.

III. The Growth of P. hominis, Strain Huf-2, in Varied Population Levels of E. coli in a Glucose-Salts Synthetic Medium.

Previous experiments indicated that the growth of P. hominis, strain Huf-2, was only moderately affected by most of the bacteria. However, this was in a nutritionally complete medium for P. hominis. To test the ability of E. coli to support the growth of P. hominis, a glucose-salts synthetic medium was used which would by itself support the growth of the bacteria but not the trichomonads.

Materials and Methods. The inoculant culture of E. coli was grown for 24 hours in the glucose-salts synthetic medium. An inoculum of 0.1 ml was introduced into each tube of 4 series of 4 tubes each containing glucose-salts synthetic medium. Tubes in the first series contained one unit; tubes in the second series, 5 units; tubes in the third series, 10 units; and tubes in the fourth series, 20 units per ml of both penicillin and streptomycin. A fifth series of tubes received no antibiotics or bacteria. The bacteria were given two hours to adjust to the medium and then a suspension of P. hominis in glucose-salts synthetic medium was inoculated into the growth tubes. The experiment was terminated at 34 hours by taking a pH reading and counting the bacterial and trichomonad populations.

Results. As seen from Fig. 2, the antibiotics allowed varying levels of bacteria to develop throughout the experiment. The tri-

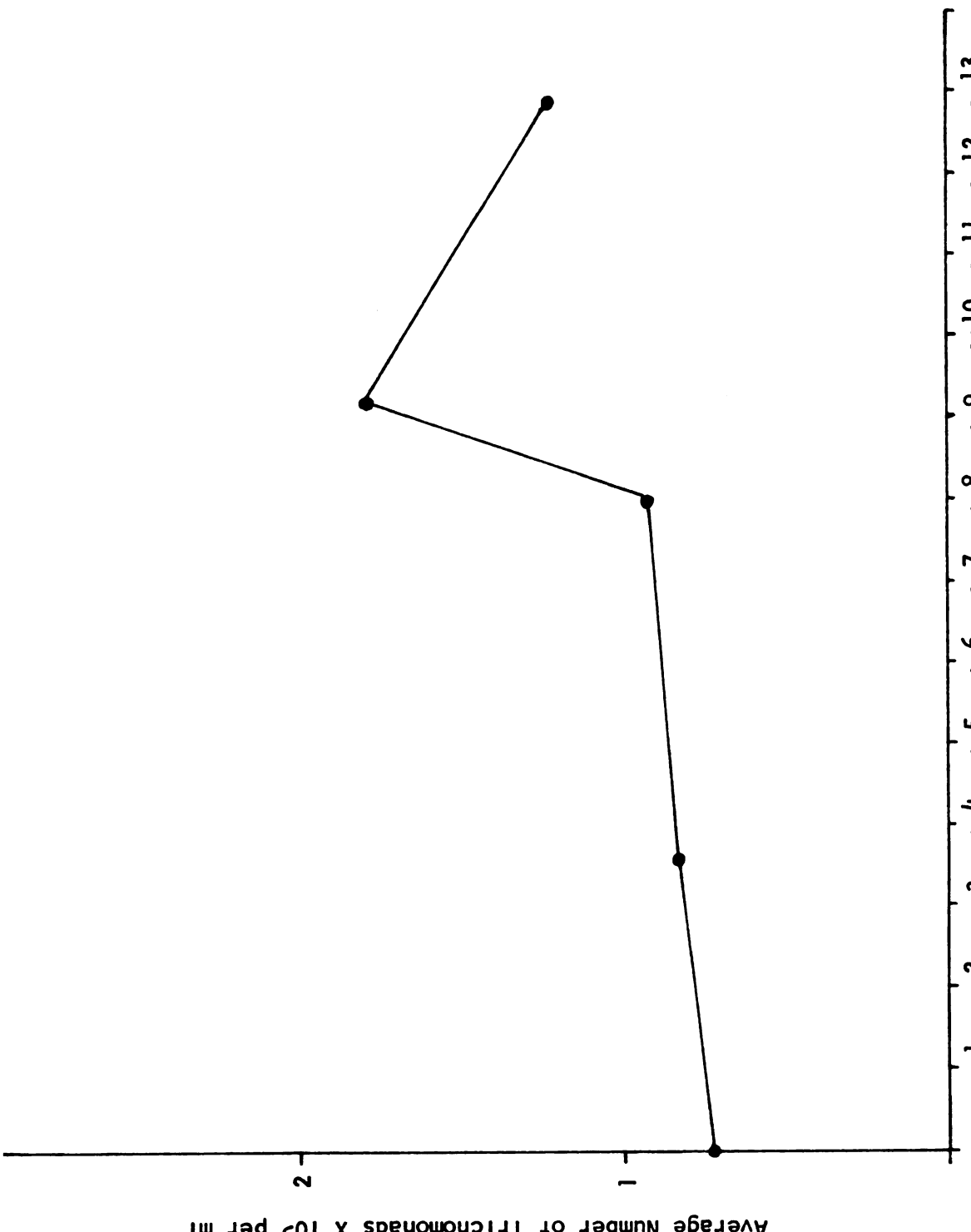

FIG. 2. Growth of P. hominis, Strain Huf-2, in Four Population Levels of E. coli in Glucose-Salts Synthetic Medium.

chomonads appeared to grow best with about 10^9 E. coli per ml. With an E. coli population of 10^8 organisms per ml, the number of trichomonads was significantly different from the bacteria-free control at the 5% level of probability and with bacterial levels of 10^9 and 10^{13}/ml they were significant at the 1% level.

Discussion and Conclusions. The results indicated that an optimum concentration of bacteria did support growth slightly higher than the bacteria-free control. It was difficult to imagine that this minimum growth over the controls, no more than a 2-fold increase in numbers, would sustain the trichomonads over several cultural transfers. It seemed doubtful if the bacteria supplied all the essential nutrients needed by P. hominis.

IV. The Constituents of Modified Diamond's Medium Essential for the Growth of Two Strains of P. hominis with E. coli.

Since P. hominis showed very poor growth with E. coli in a deficient medium, an attempt was made to determine what components of modified Diamond's medium were necessary for growth in a trichomonad-E. coli association. In order to accomplish this, major components of modified Diamond's medium were eliminated singly from the medium.

Materials and Methods. The general procedures used in experiments 1, 2, and 3 were similar. Separate series of tubes of media were prepared. The media of each series lacked one of the four major components of Diamond's medium (trypticase, yeast extract, glucose, or serum). Within each series 4 to 5 subseries of 4 to 5 tubes each were prepared without antibiotics as a bacteria-free control. The inoculum culture of E. coli was grown in glucose-salts synthetic medium for 24 hours.

The trichomonads were suspended in Kreb's Ringer's solution which was added in 1 ml quantities to each tube to give a final concentration of 1.0×10^5 trichomonads per ml.

The time for terminating the three experiments varied. Experiments 1, 2, and 3 were terminated at 24, 30, and 31 hours, respectively. Bacterial counts were only made on the first experiment. The pH was taken before the tubes were killed and the trichomonads counted.

In each series with different levels of antibiotics, the bacteria-trichomonad subseries with the highest trichomonad population was chosen to compare with bacteria-free controls.

Results. The results are given in Table II. Throughout the experiments the pH did not seem to cause any noticeable effect on trichomonad numbers. The difference between the two strains was brought out in this experiment by their different abilities to utilize E. coli in place of the missing medium component. Strain 5MS produced significantly higher populations with bacteria than without bacteria when trypticase, yeast extract, glucose, or serum was deleted from modified Diamond's medium, while strain Huf-2 showed no higher population with bacteria when trypticase or serum was deleted. The strain Huf-2 was able to utilize the bacteria for part of its glucose requirement, but even here strain 5MS was able to use E. coli to a much greater extent than strain Huf-2.

Discussion and Conclusions. At optimum growth with E. coli the 5MS strain of P. hominis was able to utilize the bacteria to replace trypticase, yeast extract, or serum. The growth of P. hominis, 5MS strain, in the absence of serum and yeast extract was not high, but it

TABLE II. A Comparison of the Growth of Two Strains of *P. hominis* with *E. coli* in the Partial Components of Modified Diamond's Medium.

Exp.	Strain	Bacteria/ml or Pen.-Strep./ml	Nutritional Components				Trichomonads X 10^5/ml*				F value
			Trypti-case %	Yeast Extract %	Glucose %	Serum %	With *E. coli* ± S.E.	pH	Without *E. coli* ± S.E.	pH	
1	Huf-2	$2.0x10^{11}$	-	1	0.5	5	1.32 ± .05	6.1	1.43 ± .04	6.7	3.57
1	Huf-2	$1.2x10^{6}$	2	1	-	5	9.05 ± .17	6.6	7.29 ± .18	6.7	50.53**
1	Huf-2	$2.4x10^{15}$	2	1	0.5	-	1.57 ± .04	5.3	1.42 ± .07	6.8	3.31
2	5MS	10 units	2	1	0.5	-	1.85 ± .10	-	1.15 ± .10	-	24.97**
2	5MS	10 units	2	1	-	5	13.46 ± .18	-	5.64 ± .14	-	1181.30**
3	5MS	3 units	-	1	0.5	5	4.36 ± .10	6.4	1.47 ± .05	6.6	683.41**
3	5MS	3 units	2	-	0.5	5	2.80 ± .08	6.6	1.45 ± .05	6.7	193.88**

* Each mean based on 4 tubes.

** Significant at the 1% level of probability.

was at least significantly higher than the growth in the bacteria-free controls. On the other hand, the Huf-2 strain of P. hominis could not grow with bacteria in the absence of trypticase or serum. Glucose was not an essential supplement in the presence or absence of E. coli with either strain although strain 5MS of P. hominis was able to utilize the bacteria for their energy requirements to a much greater degree than strain Huf-2.

Since these experiments were terminated at a given time and there were no subcultures, the ability of strain 5MS to use E. coli for sustained growth will be evaluated in a later experiment.

V. The Growth of P. hominis, Strain 5MS, with E. coli in Glucose-Salts Synthetic Medium Enriched with Supplemental Nutrients.

P. hominis, strain 5MS, was used throughout the remainder of the research because of its greater ability to utilize E. coli for nutrients than strain Huf-2. The strain 5MS was recently isolated and therefore probably retained more of its in vivo capabilities to use bacteria.

It was felt that a better understanding of the nutrients which the bacteria were supplying to P. hominis could be accomplished by growing the trichomonads with bacteria in a medium which supports E. coli, but which contains few or none of essentials for trichomonad nutrition. The glucose-salts synthetic medium seemed suitable for this purpose since it supported the growth of E. coli but supplied only energy requirements to the trichomonads. To this medium sources of known trichomonad nutrients were supplied in various combinations to determine what nutrients the bacteria failed to supply the trichomonads.

Materials and Methods. The glucose-salts synthetic medium, sup-

plemented with metal mix #50 (Lee and Pierce, 1960) as a source of trace elements, served as the basic medium for each of the experiments. The effects of the following nutritional supplements were tested in the following concentrations: 2 g% trypticase, 1 g% yeast extract, 5% v/v calf serum, 2% v/v or double strength vitamin mix #12 (Hutner, et al., 1957), 80 mg% alkali-hydrolized yeast RNA (N.B.Co.), 0.5 mg% cholesterol, and 1.0 mg% TEM 4T (Hachmeister - Inc.). Each supplement was added to concentrated glucose-salts synthetic medium. The final dilution with the above supplements, the trichomonads, and water brought the glucose-salts synthetic medium and all supplements to the described concentration.

E. coli inoculants came from cultures raised for 12 hours in glucose-salts synthetic medium. The trichomonads were suspended in an aliquot of the glucose-salts synthetic medium for inoculation into the experiments. Low concentrations of penicillin and streptomycin were used in the experiments to slow bacterial growth.

Changes of pH were observed by the use of brom cresol purple indicator in the medium. After 28 hours of growth the experiments were terminated. Counts of the bacterial populations were only made in experiment 3.

Results. Experiment 1 and part of experiment 3 represented attempts to determine if P. hominis would grow in the presence of E. coli, the glucose of the basic medium, and serum.

The first two lines of Table III showed that there was no appreciable growth over the inoculum with serum alone as the growth supplement. The bacteria-free control had a population of 0.93×10^5

trichomonads per ml. The remaining data on Table III showed that either trypticase and yeast extract together or RNA alone would support trichomonad growth when added to the basic medium supplemented with serum. Yeast extract was undoubtedly a source of RNA.

Evidently the vitamin requirements were obtained either from the bacteria or the serum, since the addition of vitamins to the RNA did not enhance growth over that obtained with RNA alone.

Various authors have suggested the presence of unknown growth factors in trypticase and serum (Sanders, 1957; Shorb and Lund, 1959; Lee and Pierce, 1960). In an attempt to determine if E. coli could supply the serum factor and the trypticase factor in the absence of serum, vitamin mix #12, cholesterol, and TEM 4T (a source of fatty acids) were substituted for serum in part of experiment 3. The latter two ingredients have been shown to be growth requirements for trichomonads and some vitamins are probably essential. The results are shown in Table IV. The addition of RNA, vitamin mix, cholesterol and TEM 4T to the basic glucose-salts medium did not support trichomonad growth in the presence of E. coli. The addition of trypticase to the same mixture of supplements gave good growth, but the addition of yeast extract without trypticase did not support growth. It is interesting to note that in Experiment 1 on Table III, under almost similar conditions, serum supplied an unknown growth factor or factors in the absence of trypticase. It appears that possibly serum and trypticase have an essential factor or essential factors which, if not the same, are similar enough to support the growth of P. hominis under certain nutritional conditions.

Using trypticase, metal mix, and RNA as supplements in the glucose-salts medium, the ability of E. coli to supply the vitamin, cholesterol, and TEM 4T factors was tested in Experiments 2 and 4.

The results of Experiment 2 and the first group of Experiment 4 in Table V confirms previous observations that vitamins, cholesterol and TEM 4T would not support the growth of P. hominis in bacterial cultures in the absence of trypticase. Trypticase without vitamins, cholesterol or TEM 4T also failed to support growth. Vitamins and TEM 4T, vitamins and cholesterol, and cholesterol and TEM 4T were tested with trypticase as combinations. Trichomonads grew only where cholesterol was included in the medium. Cholesterol appeared to be a necessary nutrilite for the trichomonads along with the unknown trypticase-serum factor and RNA that E. coli could not supply to P. hominis.

Discussion and Conclusions. Cholesterol appeared to be a necessary nutrilite for the trichomonads in the presence of E. coli in addition to RNA and the unknown requirements met by either serum or trypticase. The effect of metals was not tested since a highly purified medium would be essential to eliminate trace mineral contaminants and thus show what metals were required.

TABLE III. The Effect of Supplemental Nutrients on the Growth of *P. hominis*,[a] Strain 5MS, with *E. coli* in a Glucose-Salts Synthetic Medium[b] plus Serum.

Exp.	Bacteria/ml or Pen.-Strep./ml	Supplemental Nutrients				No. of tubes	Trichomonads X 10^5/ml ± S.E.[d]
		Trypticase %	Yeast Extract %	Alkali-hyd. Yeast RNA mg %	Vit. Mix[c] ml/100		
1	5 units	-	-	-	-	4	1.37 ± .03
3	10^3-10^8	-	-	-	-	4	1.12 ± .05
1	5 units	2.0	1.0	-	-	4	6.76 ± .11
1	5 units	-	-	80.0	2.0	4	6.24 ± .10
3	10^{12}	-	-	80.0	2.0	4	3.77 ± .04
3	10^{12}	-	-	80.0	-	4	3.79 ± .04
3	10^3-10^8	-	-	-	2.0	4	1.24 ± .03

[a] At 28 hrs.

[b] With the addition of Metals Mix No. 50 (Lee and Pierce, 1960).

[c] Vitamin Mix No. 12 (Hutner, *et al.*, 1957).

[d] Exp. 1: inoculated with 1.10×10^5 trichomonads/ml.
Exp. 3: inoculated with 1.15×10^5 trichomonads/ml.
Bacteria-free control - 0.93×10^5 trichomonads/ml.

TABLE IV. The Effect of Supplemental Nutrients on the Growth of *P. hominis*,[a] Strain 5MS, with *E. coli* in an Enriched[b] Glucose-Salts Synthetic Medium.

Exp.	Bacteria Level	Supplemental Nutrients		No. of tubes	Trichomonads X 10^5/ml[c]
		Trypticase %	Yeast Extract %		
3	10^4/ml	-	-	4	0.85 ± .04
3	10^7-10^{10}/ml	2.0	-	4	4.31 ± .08
3	10^{10}-10^{12}/ml	-	1.0	4	0.78 ± .04
3	10^{13}/ml	2.0	1.0	4	2.56 ± .07

[a] At 28 hrs.

[b] Metal Mix No. 50 (Lee and Pierce, 1960), 6.5 mg %; Vitamin Mix No. 12 (Hutner, *et al.*, 1957), 2 ml/100; Tem 4T, 1 mg %; cholesterol, 0.5 mg %; and RNA, 80 mg %.

[c] The inoculum was 1.15×10^5 trichomonads/ml.

TABLE V. The Substitution of Various Nutrients for Serum in the Growth[a] of *P. hominis*, Strain 5MS, with *E. coli* in an Enriched Glucose-Salts Synthetic Medium.[b]

Exp.	Pen.-Strep./ml	Supplemental Nutrients				No. of tubes	Trichomonads X 10^5/ml[d]
		Trypticase	Vit. Mix[c]	Cholesterol	TEM 4T		
		%	ml/100	mg %	mg %		
2	5 units	-	2.0	0.5	1.0	4	1.24 ± .06
2	5 units	2.0	2.0	0.5	1.0	4	4.37 ± .07
4	6 units	2.0	2.0	0.5	1.0	4	8.09 ± .13
4	6 units	2.0	2.0	-	-	4	0.59 ± .06
4	6 units	2.0	2.0	-	1.0	4	0.42 ± .03
4	6 units	2.0	2.0	0.5	-	4	13.40 ± .11
4	6 units	2.0	-	0.5	1.0	4	7.10 ± .27

[a] At 28 hrs.

[b] With the addition of Metals Mix No. 50 (Lee and Pierce, 1960), 6.5 mg %; and alkali-hydrolyzed yeast RNA, 80 mg %.

[c] Vitamin Mix No. 12 (Hutner, *et al.*, 1957).

[d] Exp. 2: inoculated with 0.86×10^5 trichomonads/ml.
Exp. 4: inoculated with 0.98×10^5 trichomonads/ml.

VI. Sustained Growth of *P. hominis*, Strain 5MS, with *E. coli* in a Minimum Medium.

In most of the previous experiments, *P. hominis* did not achieve high populations and it is possible that it might take several generations for the absence of nutritional factors to stop growth. The objective of this experiment was to see whether the ability of the bacteria to substitute for various nutritional factors could be sustained over a series of transfers.

Materials and Methods. The basic medium used in this experiment was the glucose-salts synthetic medium supplemented with 2 g% trypticase and 6.5 mg% metals mix #50 which served as a control. The experimental medium consisted of the basic medium with cholesterol and alkali-hydrolized yeast RNA added separately and in combination.

Each lot described above consisted of twelve to fifteen 5 ml tubes. The tubes were autoclaved and then stored under refrigeration. There was no allowance made for dilution with the inoculum.

The growth of the bacteria and trichomonads was followed by observing changes in the brom cresol purple indicator and by microscopic examination. Penicillin-streptomycin solution was added as needed to the cultures to keep the bacteria population from overgrowing the medium. When growth seemed to reach its maximum, a 0.5 ml inoculum was transferred to a fresh tube.

Transfers served to inoculate both trichomonads and bacteria. A hemocytometer count of the trichomonads was made only at the end of the last transfer. No attempt was made to determine the bacterial populations.

Results. The general time between transfers varied from 36 to 96 hours depending upon the trichomonad-bacteria balance. There was always difficulty in balancing both bacteria and trichomonad numbers to obtain optimum growth of the trichomonads.

Table VI shows that the suggested minimum medium for growth on a single transfer in the presence of E. coli consisting of glucose-salts medium, trypticase, cholesterol, and RNA, supported trichomonad growth for 7 transfers in the presence of bacteria and supported no growth when E. coli was absent.

When cholesterol was deleted from the minimum medium, E. coli was unable to sustain growth of the trichomonads for even a single transfer, but when RNA was deleted from the medium the trichomonads were able to utilize E. coli for the RNA factor over 3 transfers at which time the experiment was terminated.

Discussion and Conclusions. The observations of the previous section were confirmed. Trypticase, cholesterol and RNA supplements added to an E. coli population would support growth of P. hominis. In addition, the successive transfer experiments suggest the RNA factor can be satisfied by living bacterial cells. It was possible to culture the trichomonads through a series of three transfers with E. coli in a minimum medium lacking supplemental sources of RNA (Table VI). This was contrary to the results employing a single passage with E. coli (Table III). Thus E. coli served as a source of alkali-hydrolized yeast RNA. The low growth of P. hominis without RNA on a single transfer (Table III) suggested either that the optimum level of bacteria was not present or that the antibiotics interfered with the ability of the bacteria to meet

the optimum nucleic acid requirements of the trichomonads. Although final populations of the trichomonads with and without RNA (Table VI) were low, the serial transfers represented a 1000-fold dilution of the inoculum.

The lower populations of the trichomonads in the serial transfer of *P. hominis* (Table VI) at the end of the sustained growth period probably resulted from the difficulty in achieving an optimum level of bacteria in an experiment of this sort.

TABLE VI. Sustained Growth of *P. hominis*, Strain 5MS, in a Minimum Medium.[a]

Exp.	*E. coli*	Cholesterol	Alkali-hyd. Yeast RNA	No. of Successful Transfers	Trichomonads X 10^5/ml[b] at Termination of Experiment
		mg %	mg %		
1	Present	0.5	80	7+	1.62
2	Present	0.5	80	3+	1.20
2	Absent	0.5	80	0	0
1	Present	-	-	0	0
2	Present	-	-	0	0
2	Present	-	80	0	0
2	Present	0.5	-	3+	0.89

[a] Minimum medium has a base of Glucose-Salts Synthetic Medium, 6.5 mg % Metals Mix No. 50; and 2.0% Trypticase.

[b] The inoculum was 1.8×10^5 organisms/ml for Exp. #1 and 1.04×10^5 organisms/ml for Exp. #2.

GENERAL DISCUSSION AND CONCLUSIONS

Bacteria seem to have the ability to supply trichomonads with a wide spectrum of nutritional products. In the intestine it is possible that the trichomonads rely on the bacteria for many of their nutrients, and where there is a wide range of bacterial species, there may be forms which supply more growth requirements than *E. coli*.

Trichomonad populations raised with most bacteria in complete medium did not reach the same numbers as those cultured axenically. On the other hand, bacterial filtrates in the culture medium did not inhibit the growth of the trichomonads, suggesting the absence of strong toxins. Although it is evident that excessive bacterial populations would inhibit growth of trichomonads, it is more easily explained by a depletion of nutrients and production of a generally unfavorable ecological environment than by a specific toxin affecting the trichomonads. Thus the results of this research tend to confirm the conclusions of Pray (1952) and to disagree with Johansson, *et al.* (1947) who suggested a strong toxic inhibition with both live bacteria of most species and their corresponding cell-free filtrates.

Although there was no evidence in this investigation of the bacteria exerting a toxic effect on the trichomonads, the bacterial contribution to trichomonad growth is a complex phenomenon yet to be completely analyzed.

The 5MS strain of *P. hominis* was able to utilize *E. coli* to replace trypticase, glucose, yeast extract, or serum in modified Diamond's medium. *P. hominis*, strain Huf-2, could not use *E. coli* as a source of

the nutritional factors found in either serum or trypticase. Strain Huf-2 did utilize the bacteria to replace glucose but not as well as strain 5MS. These experiments did indicate that the bacteria can supply many growth factors to some trichomonads, but that P. hominis, strain Huf-2, probably has lost its ability to utilize the bacteria to any extent in its nutrition.

The work of Kupferberg, Johnson, and Sprince (1948) suggested that there were unknown growth factors in trypticase and serum. The more definitive studies of Shorb and Lund (1959) and Lee and Pierce (1960) revealed that cholesterol and TEM 4T, as a source of fatty acids, could be used to replace serum in media; and that vitamin mix, alkali-hydrolized yeast RNA, and metals mix substituted for yeast extract. Thus these five supplemental nutrients could be used in the replacement of the grossly undefined yeast extract and serum.

Shorb and Lund (1959) also noticed unknown growth factors in trypticase, but they did not attempt to show if the factors were common to both serum and trypticase. The results of this work (Tables III and IV) demonstrate the presence of similar unknown growth factor in both serum and trypticase. This suggests that either ingredient may satisfy the requirement, since either serum or trypticase but not both were essential for the growth of P. hominis with E. coli.

It seems probable that E. coli or its metabolites were able to substitute for the bulk of the amino acids required in the nutrition of P. hominis since they grew with serum as the only undefined constituent of the medium (Table III). Since serum contains a low concentration of free amino acids and trichomonads seem to be unable to digest

proteins (Lwoff, 1951; Weiss and Ball, 1947), it seems doubtful that P. hominis is able to meet any substantial amino acid requirements from the low concentrations of serum used. It has been demonstrated that the alkali-hydrolized yeast RNA may have impurities which contribute at least peptides to the nutrition of trichomonads (Shorb and Lund, 1959), but it is doubtful if the entire amino acid requirements of P. hominis can be satisfied by these impurities.

Besides the amino acids, E. coli was demonstrated to be able to supply the trichomonads with many vitamin and fatty acid requirements (Tables III and V) although the exact nature of the vitamins and fatty acids is not known for P. hominis.

It seems obvious from the results of this investigation that P. hominis, like other trichomonads, has a sterol requirement and that the bacteria are unable to supply this requirement. Cailleau (1939) demonstrated that killed Shigella and even living bacteria in contaminated cultures could not replace the sterol requirement of T. batrachorum. Gunsalus and Stanier (1960) in reviewing the lipid nature of bacterial cells concluded that the presence of sterols in bacterial cells has remained largely unconfirmed.

In the sustained growth experiment (Table VI) it was found that trichomonad growth with bacteria in the absence of RNA was low and that the low growth was maintained to the termination of the experiment. As stated previously this low population could be due to a poor trichomonad-bacteria balance or to interference with the bacterial RNA metabolism.

Streptomycin was probably the effective antibiotic in controlling the populations of E. coli, since penicillin is ineffective against

most of the gram negative bacteria. Peretz and Polglase (Welch and Marti-Ibanez, 1957) demonstrated that in E. coli streptomycin forms insoluble complexes with nucleic acids which even nucleases cannot dissolve. Robinson (1953) found that streptomycin inhibited the dephosphorylation of bacterial mononucleotides. Thus the action of streptomycin upon the bacteria might interfere with their ability to provide the trichomonads with the necessary RNA factors for growth.

The observations of Hegner (1923, 1924, 1937), that the disappearance of trichomonads from the intestine was due to the toxic products of proteolytic bacteria which resulted from the host's heavy protein diet, does not seem very probable in the light of this research and that of Pray (1952). Living proteolytic bacteria such as E. coli, P. vulgaris and C. sporogenes as well as the filtrates of four bacterial species had no demonstrable toxic effect upon the trichomonads in modified Diamond's medium.

Possible factors which could bring about changes of trichomonad populations in the intestine with differences in diet and bacteria could be: (1) changes in the physical environment of the intestine such as varied intestinal volume, viscosity and pH, (2) differences in the intestinal secretions, and (3) differences in the relative ability of different bacteria to supply the trichomonads with growth factors.

This research has demonstrated that the relationship between trichomonads and bacteria could be a very close one. At least in vitro the bacteria seem able to serve as sources of much of the trichomonads nutritional requirements.

Promising future areas of research in the study of bacterial-trichomonad relationships, as suggested by this work are: (1) the use of more definitive *in vivo* observations; (2) the use of more bacteria, especially of the strict putrifactive and acidophilic type, in determining the ability of different species to support growth of trichomonads *in vitro*; and (3) the study of *in vitro* relationships of other intestinal members of the Trichomonadidae with bacteria.

SUMMARY

1. *Pentatrichomonas hominis*, strain Huf-2, was cultured in a complete medium with various bacterial species and in the presence of cell-free bacterial filtrates. No evidence of bacterial-induced toxin inhibition was noticed. In fact, cell-free preparations were shown to have a slight stimulatory effect upon the trichomonads. There was a decrease in growth of *P. hominis* with most living bacteria probably explained by the competition for available nutrients.

2. Modified Diamond's medium with essential growth components eliminated was used to test the relative ability of *P. hominis*, strains Huf-2 and 5MS, to utilize *Escherichia coli* for the missing essential component. *P. hominis*, strain 5MS, was able to utilize *E. coli* to replace trypticase, yeast extract or serum although the trichomonad growth in the absence of yeast extract or serum was much lower than with the deletion of trypticase. *E. coli* could not substitute for either trypticase or serum when the Huf-2 strain was used.

3. A glucose-salts synthetic medium, which was demonstrated to be non-supportive for *P. hominis*, was enriched with varying combinations of trypticase, alkali-hydrolized yeast RNA, vitamin mix, cholesterol, and TEM 4T. The ability of E. coli to replace these supplements in the nutrition of *P. hominis* was tested, and *P. hominis* was found to be able to use *E. coli* to replace all of the supplements except alkali-hydrolized yeast RNA, a factor or factors in serum and trypticase, and cholesterol. Thus it seems that the trichomonads utilized *E. coli* as a source of the bulk of the amino acids, most vitamins, and long chain fatty acids.

4. It was demonstrated that the trypticase and serum had common unknown growth factors which did not permit the simultaneous deletion of serum and trypticase from the medium.

5. In determining the ability of *E. coli* to sustain growth of *P. hominis*, strain 5MS, over a series of 3 to 7 transfers in a glucose-salts trypticase medium with varying combinations of alkali-hydrolized yeast RNA, vitamin mix, cholesterol and TEM 4T, it was found that cholesterol was the only essential supplement. Thus *P. hominis*, strain 5MS, was also demonstrated to be able to use *E. coli* as a source of RNA.

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