A COMPARATIVE STUDY OF CULTURAL, MORPHOLOGICAL AND SEROLOGICAL ASPECTS OF THREE TRICHOMONAD SPECIES

By

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A THESIS

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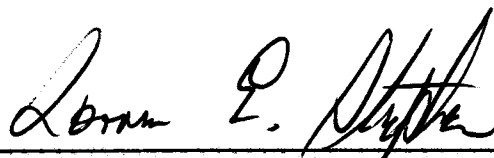degree of
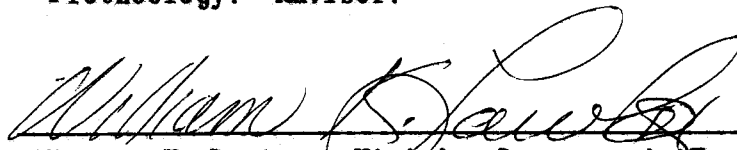
MASTER OF SCIENCE IN

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September 1968

A COMPARATIVE STUDY OF CULTURAL, MORPHOLOGICAL

AND SEROLOGICAL ASPECTS OF THREE

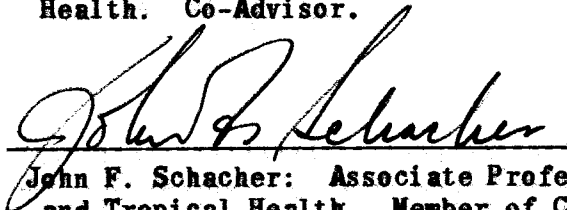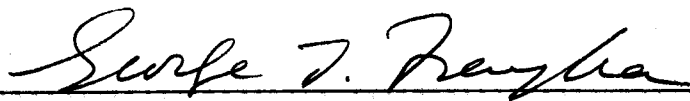## TRICHOMONAD SPECIES

By

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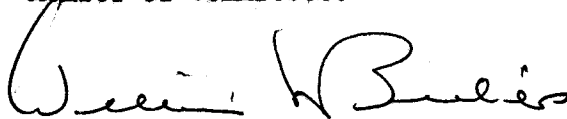
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THREE TRICHOMONAD SPECIES

KHOURY

ACKNOWLEDGEMENTS

AN ABSTRACT OF THE THESIS OF

Philip B. Khoury for Master of Science in Tropical Health
                    Major: Parasitology

Title: A comparative study of cultural morphological and
        serological aspects of three trichomonad species.

        Tritrichomonas foetus, Trichomonas gallinae and Trichomonas
vaginalis were used as parasite strains. They were employed in
evaluating four culture media: Feinberg's medium, Diamond's medium,
C.P.L.M. medium and fluid thioglycollate medium. Feinberg's medium
proved to be the best as evidenced by growth rate studies performed
on the trichomonads; T. gallinae exhibited highest multiplication
and T. vaginalis lowest.

        Six staining methods, Chlorazol Black E, Heidenhain's iron
hematoxylin, Giemsa's stain, Wright's stain, Papanicolaou's stain and
Best's Carmine, were evaluated for their ability to stain the
organelles of the trichomonads. Chlorazol Black E demonstrated best
the nucleus and the cell membrane whereas Heidenhain's iron
hematoxylin and Wright's stain were the best to demonstrate the
organism as a whole.

        Micro-agar-gel immunodiffusion, direct precipitin and
agglutinin tests demonstrated the following:

1. Lyophilized trichomonad antigens stimulated a higher antibody
    response than live ones.

2. Tr. foetus was a stronger antigen than T. gallinae which in its
    turn was stronger than T. vaginalis.

3. Anti-T. gallinae sera contained more shared antibodies to T.
    vaginalis than to Tr. foetus.

4. Anti-Tr. foetus sera contained more shared antibodies to T.
    vaginalis than to T. gallinae.

5. Anti-T. vaginalis sera contained more shared antibodies to Tr.
    foetus than to T. gallinae.

6. Natural antibodies, agglutinins or precipitins to trichomonad
    antigens were absent in normal rabbit sera and in the sera of
    three infected human patients and three infected pigeons tested.

7. Soluble- or exo-antigens from the three trichomonads were present in culture media after separation of the cellular organisms by centrifugation.

The three trichomonads are antigenically related and they have a "common antigenic pool" which is probably generic in nature. Only T. gallinae, by the use of absorption tests, was shown to have a specific antigen which it does not share with Tr. foetus or T. vaginalis.

Tr. foetus and T. vaginalis are almost serologically indistinguishable.

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I. INTRODUCTION

Trichomonas Donné, 1837 and Tritrichomonas Kofoid, 1920 are two protozoan genera belonging to the family Trichomonadidae within the order Trichomonadorida (Levine, 1961). Members of these genera are mainly differentiated on the basis of the number of anterior flagella present; members of Trichomonas possess four anterior flagella whereas members of the Tritrichomonas possess three anterior flagella.

For the last five decades, many workers have been engaged in studying the various species belonging to the above genera; these workers were concerned with the cultivation, morphology, physiology, histochemistry, pathogenicity, chemotherapy and immunology of these organisms. The main bulk of the work done was on the maintenance and cultivation of the parasites. Trichomonas vaginalis, has been cultivated in many different types of liquid media. At first simple bacteriological media were used (Lynch, 1915, for example). Next more specific culture media were put forward, C.P.L.M. (Johnson and Trussell, 1943), simplified trypticase medium (Kupferberg et al., 1948), a casein hydrolysate-serum medium (Lash, 1950) and Diamond's medium (Diamond, 1957). In addition to culturing T. vaginalis, Diamond (Diamond, l.c.) established axenic cultures of T. gallinarum, T. eberthi, T. gallinae, T. batrachorum, T. foetus and T. hominis; all these grew at 35.5°C, except

T. gallinarum which grew better at 38.5°C. Feinberg and
Whittington (1957) devised a culture medium for T. vaginalis and
Candida, which was reported as being "cheap, easy to prepare and
use, and keeps well". McEntegart (1954) maintained in his
laboratory, T. vaginalis, T. foetus (Belfast), T. gallinae and
T. hominis. The first three species reached their peak of growth
about 18 to 20 hours after inoculation; T. gallinae gave the highest
counts while T. vaginalis and T. hominis the lowest. Honigberg
(1961) used BBL fluid thioglycollate medium for the maintenance of
three strains of T. gallinae and three strains of T. vaginalis;
growth curves of these strains demonstrated that, unlike those of
T. gallinae, the strains of T. vaginalis exhibited growth rates
in vitro that were inversely proportional to their virulence in the
experimental hosts (mice). Recently, Samuels (1965) developed a serum-
free medium for the growth of axenic trichomonads: this medium
"meets the requirements of: (1) absence of foreign protein for
immunologic work, (2) saving of serum cost and one operation, and
(3) less danger of contamination in handling".

There have been few reports of growth of T. vaginalis on
solid media. Wirtschafter (1954) described a culture overlay
method of maintaining T. vaginalis on slants for 20 or more days.
Asami et al. (1955) described methods of growing the organism on media
in Petri plates and also in stab cultures. Filadora and Orsi (1958)
described methods of growing the organism on the surface of media or
by a pour plate technique. Ivey (1961) described a convenient pour
plate technique for obtaining isolated clones of T. vaginalis; he

found that under anaerobic conditions clones of the organisms developed into colonies ranging from 0.2 to 2.0 mm in size in about five days; he found also that prolonged exposure of organisms to oxygen and/or 25^{0}C proved detrimental to clone development. Samuels and Stouder (1960) grew T. vaginalis, T. gallinae and T. gallinarum, Pentatrichomonas hominis, and Tritrichomonas augusta, T. foetus, and T. suis, in agar pour plates of C.P.L.M. and Diamond's media; they obtained visible colonies in two to five days of partially anaerobic incubation with added CO_2, depending on the species used.

Apart from using liquid and/or solid media, freezing was used by many workers to maintain trichomonads. Weinman and McAllister (1947) demonstrated that a number of pathogenic protozoa would survive freezing and thawing and could be stored in the frozen state without deteriorating. Amongst those successfully preserved were trypanosomes, leishmania and plasmodia. The observations of these workers on trichomonads were limited to two species, T. vaginalis and T. hominis, both cultivated with bacteria as a nutrient. Of these T. vaginalis failed to survive at all and T. hominis was preserved in a proportion of the experiments only. During an investigation into the survival of trichomonads at -79^{0}C in the presence of glycerol (McEntegart, 1954) it was noticed that, of four species tested (T. vaginalis, T. foetus, T. gallinae and T. hominis), T. foetus was the only one which failed to survive freezing. The same observation was made independently by Joyner and Bennett (1956) who observed that freezing to -79^{0}C in the presence of glycerol was a method of destroying contaminating T. foetus in bull

semen. On the other hand Levine and Marquardt (1954) record that the addition of five to ten percent glycerol enabled T. foetus to survive when frozen and stored at $-79^{\circ}C$.

Finally, Honigberg et al. (1965) were able to preserve T. vaginalis and T. gallinae in liquid nitrogen in the presence of dimethyl sulfoxide. They noted that the average survival rates of the five strains of T. vaginalis and of a single one of T. gallinae were about 80 to 100 percent, after five to eight months of storage at $-196^{\circ}C$.

Many studies have been made on the morphology of trichomonads employing conventional protozoal staining techniques. Very few workers, however, have studied the value of various stains for selectively demonstrating the various organelles. The morphology of T. vaginalis has been described in great detail, both from stained specimens and from living cells examined by phase contrast microscopy. Bauer et al. (1959) studied the cytology of vaginal smears using Papanicolaou's staining technique (Papanicolaou, 1954). They found that "a typical cell features, leucocytic clusters, ground-glass appearance, and the presence of long bacillary forms in chains" were indicative of trichomoniasis infection, but they did not demonstrate the parasite with this technique. Hayes and Kotcher (1960) examined specimens of vaginal secretions for T. vaginalis by (1) wet preparation, (2) Papanicolaou's staining technique, (3) culture in sodium thioglycollate, and (4) fluorescent antibody techniques. Of the 225 women examined 33.3%, 40%, 13%, and 39.6% were positive for T. vaginalis by wet preparation, culture in

artificial medium, Papanicolaou's stain and fluorescent antibody,
techniques, respectively. Ciucă and Mihalache (1961) demonstrated
that dead parasites stained with 1:1,000 solution of methylene
blue but none of the living trichomonads took up the stain. Thin
air-dried _Trichomonas_ smears were satisfactorily stained for four
to five minutes with Gentian violet, basic fuchsin, methylene blue,
alcohol and physiological saline by Zhuravskii (1962). Mandal _et al_.
(1963) stained _T. foetus_ smears, after fixation by osmic acid vapour
bringing out all the morphological details. Jensen and Hammond
(1964) used Bodian's technique (Honigberg and Davenport, 1954),
Heidenhain's iron hematoxylin (Kudo, 1954) and a Giemsa staining
method for blood smears (Kudo, l.c.), to study the morphology of
trichomonads and related flagellates from the bovine digestive tract.
Recently, the structure of _T. vaginalis_ was described more fully by
Honigberg and King (1965) by studying 12 strains of the species
on the basis of observations of living material with the aid of phase
contrast and fixed preparations stained with iron hematoxylin (or
hematein), and protargol. Amies and Garabedian (1965) used fixative
slides- slides dipped in a solution of mercuric chloride and sucrose
in 30% alcohol and allowed to dry - for the diagnosis of _T. vaginalis_.
Their stained smears demonstrated the nucleus, flagella and
cytoplasm of the trichomonads which was satisfactory for diagnostic
purposes, but other morphological details were not clearly shown.
Leishman's stain was used by Lowe (1965), for the detection of
T. vaginalis; this stain satisfactorily demonstrated the flagella
and axostyle.

The fine structure of T. vaginalis was studied by Inoki
et al. (1960) who described electron micrographs of cytoplasmic
inclusions, including axostyle, Golgi apparatus, endoplasmic
reticulum and granules. They pointed out that there were
no mitochondria and related this fact to the anaerobic environment
of these organisms. Other workers have described mitochondria
in related organisms such as Tritrichomonas (Anderson and
Beames, 1959), and T. criceti (Chakraborty et al., 1961) albeit
none was found in T. foetus (Simpson and White, 1964). Smith and
Stewart (1966) by taking electron micrographs of thin sections of
osmium-and glutaraldehyde-fixed T. vaginalis cells, showed details
of the blepharoplast region, axostyle, costa, parabasal body,
endoplasmic reticulum, and chromatic granules; paracostal and
paraaxostylar granules had identical fine structure devoid of
internal membranes.

Immunological and serological aspects of trichomonads have
not been thoroughly investigated due to the fact that many early
workers found these organisms to be poor antigens. Microagglutination
experiments, however, performed by Trussell (1946) indicated that
T. vaginalis stimulated the production of antibodies in inoculated
rabbits. Sanborn (1955) using microagglutination tests also found
that T. foetus differed serologically from the large pig caecal
trichomonad, T. suis, and trichomonads isolated from pig nasal
passages. Macdonald and Tatum (1948) investigated the serological
relationships of three species of trichomonads, T. foetus, T.
vaginalis, and T. hominis. In their investigation, the immune rabbit

serum produced by the intravenous injection of formolized suspensions of protozoa was tested in two ways; (a) by observing the agglutination of similar killed suspensions, and (b) by the "agglomeration" of living suspensions of the test strains. As a result of their experiments, these workers concluded that T. foetus was antigenically related to but distinctly different from T. vaginalis and T. hominis; they considered the latter two to be antigenically identical. Menolasino and Hartman (1954) compared T. foetus and T. vaginalis, only. They used two different types of antigen to prepare immune rabbit sera; the first was a lysate of trichomonads from which all cellular debris had been removed and the second the cell residue recovered after lysis. Tests for antibody were carried out by observing microscopically the action of serial dilutions of serum on suspensions of living protozoa. These workers concluded that T. foetus and T. vaginalis were antigenically indistinguishable. McEntegart (1956) found that T. foetus var. Belfast and T. foetus var. Manley were serologically distinct.

It was not until 1960, that Robertson (1960) employed precipitin and gel-diffusion tests in Ouchterlony plates when she studied the antigens of T. foetus isolated from bovine and swine sources; she found that T. foetus var. Belfast and T. foetus var. Manley were serologically distinct, but concluded that "serological distinctions do not justify the separation of the bovine and the swine strains into two species". Recently, Johnson (1967) using micro-gel-diffusion methods (Crowle, 1961) compared six strains of T. foetus and found that they had at least four antigens

in common.

In the light of the above review of the literature, it was decided to investigate the following aspects:

1. To use _Trichomonas vaginalis_, _Trichomonas gallinae_, and _Tritrichomonas foetus_ as the parasite strains.

2. To evaluate four culture media, Feinberg's (Feinberg and Whittington, 1957), C.P.L.M. (Johnson and Trussell, 1943), Diamond's (Diamond, 1957), and fluid thioglycollate, by using them in the growth rate studies performed on the three species.

3. To evaluate six staining methods, Chlorazol Black E (Gleason and Healy, 1965), Heidenhain's iron hematoxylin (Kudo, 1954), Giemsa's stain (Shute, 1966), Wright's stain, Papanicolaou's stain (Papanicolaou, 1954), and Best's Carmine (Asami and Nakamura, 1955) for their ability to stain the different organelles of the trichomonads.

4. To compare serologically the three trichomonads using a micro-agar-gel immunodiffusion technique (Crowle, 1958, 1961), precipitin and agglutination tests (Martin and Durham, 1943), and absorption tests.

II. MATERIALS AND METHODS

Parasite Strains

Three trichomonad species were employed: <u>Trichomonas</u> <u>gallinae</u> (Rivolta, 1878) Stabler, 1938, was cultured from crop washings of locally obtained pigeons; the strain of <u>Tritrichomonas</u> <u>foetus</u> (Riedmüller, 1928) Wenrich and Emmerson, 1933, used was received from A.W. Rule, John Wyeth and Brothers Ltd., Havant, Hants., England. <u>Trichomonas</u> <u>vaginalis</u> Donné, 1836, was collected and cultured from three patients, suffering from trichomonad vaginitis and leukorrhea, seen in the Outpatient Department of the American University Hospital, Beirut.

Samples of blood were collected from the infected patients and the infected pigeons to test for the existence of natural antibodies. For control experiments blood was collected from non-infected hosts. The blood was allowed to clot and the serum was separated by centrifugation. All sera were refrigerated at $4^{0}C$, until used.

Culture Media

Four different media were used to culture the parasites. Cultures were incubated at 34^{0}- to 38^{0}-C.

1. Diamond's Medium (Diamond, 1957). The agar was omitted

from the medium and the pH was adjusted to 5.8 to 6.5 for $\underline{T.}$ $\underline{vaginalis}$ and to 6.8 to 7.2 for $\underline{T.}$ $\underline{gallinae}$ and $\underline{Tr.}$ $\underline{foetus}$. After autoclaving for 15 minutes at 15 pounds pressure, the medium was cooled to $50^{\circ}C$, and 10% inactivated horse or calf serum, potassium G, 1×10^6 units per litre, and 5×10^5 units of streptomycin sulfate per litre, were added.

2. C.P.L.M. (cysteine - peptone - liver - maltose) Medium (Trussell, 1946). After filtration 0.7 ml of 5% methylene blue solution was added per litre; the pH was adjusted to 6.0 for $\underline{T.}$ $\underline{vaginalis}$, and to 7.0 for $\underline{T.}$ $\underline{gallinae}$ and $\underline{Tr.}$ $\underline{foetus}$.

3. Feinberg's Medium (Feinberg and Whittington, 1957). Proteolyzed liver[1] 25.0 g, sodium chloride 6.5 g, and dextrose 5.0 g, were dissolved in 1,000.0 ml of distilled water. Instead of adding 80.0 ml of inactivated horse serum and then sterilizing the mixture by Seitz filtration, the mixture was sterilized in the autoclave for 10 minutes at 15 pounds pressure, cooled to $48^{\circ}C$ and then 80.0 ml of sterile inactivated calf serum were added. The pH of this medium was adjusted to 6.4 for growth of $\underline{T.}$ $\underline{vaginalis}$, and pH 7.0 for $\underline{T.}$ $\underline{gallinae}$ and $\underline{Tr.}$ $\underline{foetus}$. Initially, 1×10^6 units of penicillin and 5×10^5 units of streptomycin were added per litre; after

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1. Liver Digest. Oxoid Limited. London, S.E.1.

several subcultures had been achieved, antibiotics were discontinued.

4. Fluid Thioglycollate Medium[1]. The pH of this medium was adjusted to 6.0 for _T. vaginalis_, and 6.8 for _T. gallinae_ and _Tr. foetus_. Five percent (vol/vol) inactivated calf serum, penicillin (1×10^6 units), and streptomycin (5×10^5 units) were added after the autoclaved medium had been allowed to cool.

Growth Rate Studies

The three trichomonad species were cultured in each of the above-mentioned media, and their growth rates were determined by making 4-hourly counts over a period of 36 hours employing a Neubauer-Levy hemocytometer counting chamber.

Maintenance and Bulk Growth of the Strains

1. Tube Growth: Ten ml of medium was inoculated with 0.5 ml of an actively growing culture, incubated at 35^0- to 37^0-C and subcultured daily.

2. Bulk Growth: Ehrlenmeyer flasks containing 30 ml, 100 ml, 400 ml or three litres of medium were used. The necessary inoculum was obtained by successive 24-hours subcultures in 10 ml, 30 ml, and 100 ml of medium. The medium in each case was warmed in the incubator for three to five hours prior to inoculation.

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1. Bacto. Difco Laboratories.

Staining

To ensure that the cultures of each of the three trichomonad species were accurately identified, their morphology was examined in stained preparations under the microscope. For this purpose, the following staining methods were employed to find the most satisfactory technique.

1. Chlorazol Black E prepared by the method of Gleason and Healy (1965).

2. Heidenhain's Iron Hematoxylin (Kudo, 1954).

3. a. Giemsa's Stain. Smear's were stained as described by Shute (1966) for blood smears, except that sterile inactivated serum (1:9; serum:sample) was added to the culture aliquot prior to making smears.

   b. Wright's Stain. The same technique as used in 3. a. above, except that Wright's Stain was substituted for Giemsa's stain.

4. Papanicolaou's Staining Method (Papanicolaou, 1954). Wet films were fixed in a 1:1 solution of 95% ethanol: ethyl ether.

5. Best's Carmine (Asami and Nakamura, 1955). Dried smears were fixed in 95% ethanol before staining.

Antigen Preparation

Live Antigen

Trichomonads were collected from bulk cultures by centrifugation at $0°C$ at 2,000 rpm for ten minutes, discarding the

supernatant, and washing four times in physiological saline by
repeated resuspension and centrifugation. After the final
centrifugation the organisms were resuspended in physiological
saline and the numbers determined by counting a measured amount
in a Neubauer-Levy hemocytometer. Samples were then adjusted to
furnish a suspension containing 75 million organisms of T. vaginalis
per ml, 50 million of T. gallinae per ml and 30 million of Tr.
foetus per ml. Live antigens of all three species were thereby
adjusted to contain 1.5 mg of protein per ml.

Lyophilized Antigen

Organisms were grown and treated as above except that after
counting and adjustment of numbers of organisms, samples were
centrifuged, the organisms were resuspended in a small quantity of
distilled water, and were then lyophilized using a Freeze-Mobile,
VirTis. Samples were then stored in the refrigerator at 4°C. Before
injection into rabbits, each of the lyophilized antigens was diluted
with physiological saline to contain a standard 1.5 mg protein per
ml of solution.

Nitrogen content of lyophilized material was determined by
the micro-Kjeldahl method, using the technique of Dr. L.E. Stephen
(personal communication) and the distillation apparatus of Markham
(1942).

Preparation of Antisera

Six male rabbits, two for every trichomonad species, of
approximately the same age and weight, were used for antibody

production. After being bled for control serum-samples, rabbits I, III, and V were injected with lyophilyzed Tr. foetus, T. gallinae and T. vaginalis antigens, respectively. Live antigens of Tr. foetus, T. gallinae and T. vaginalis, were injected into rabbits II, IV, and VI, respectively.

The schedule of injections was modified slightly from that outlined by Kabat and Mayer (1964). Injections were given on alternate days. Each rabbit was given three injections of 1 ml, three injections of 1.5 ml, four injections of 2 ml, four injections of 3 ml and three injections of 5 ml. The first injection of each series was given intraperitoneally; the remaining injections intravenously. Animals were bled by cardiac puncture five and seven days after the last immunizing injection.

Micro-Agar-Gel Immunodiffusion Tests

The method used was that of Crowle (1958); 0.25% sodium azide was added to the agar to prevent bacterial growth. Tests were performed on 2- X 3- inch glass slides. Two parallel double layers of waterproof plastic electrician's tape (0.1 mm thick) was placed 5.5 cm apart along the wide edges of the slide supporting the template, which was made from 5 mm Plexiglas^{R} stock. Each template had a pair of identical well arrangements permitting two tests to be performed simultaneously. Every well arrangement consisted of a central well and six peripheral ones encircling it. Each of the peripheral wells was 6.0 mm away from the central well and the same lateral distance from its neighbour. The six peripheral

wells contained the various antigens (three live antigens and three lyophilized ones) while the central wells held the various antisera. Reactions were allowed to develop in a moist chamber for two to five days at room temperature. Reciprocally, cross-matched antigens and antibodies reacted simultaneously in order to control as many variables as possible.

At the end of the reaction period, the slides were soaked overnight in phosphate buffered saline at pH 7.4 to remove precipitated proteins. They were then placed into distilled water for ten minutes, transferred to 1% acetic acid for ten minutes for fixation of precipitin bands and then, stained for proteins, polysaccharides and lipids.

Precipitin bands were stained for lipids by Sudan Black B and Oil Red O, and Nile Blue A (Crowle, 1961). Polysaccharides were stained by Schiff Reagent (Crowle, 1961) . Alcian Blue (Heremans and Vaerman, 1958), Mayer's Mucicarmine and Basic Fuchsin (Björklund, 1954). Proteins were demonstrated by Thiazine Red R (Crowle, 1958), Corcein Scarlet MOO (Crowle, 1956), Amidoschwarz (Uriel, 1958), Azocarmine B (Kohn, 1957), Nigrosin (Kohn, 1958), Bromophenol Blue (Hayward and Augustin, 1957), and a stain composed of the following: 0.1 g Azocarmine B, 0.1 g Light Green SF, 0.1 g Naphthalene Black 10 B, 2.0 g glacial acetic acid and 0.1 g mercuric chloride, in 100 ml of distilled water; for best results the slides were kept in the staining bath for 15 to 20 minutes, and the precipitin bands were differentiated in several changes of 2% acetic acid solution (L.E. Stephen, personal

communication).

After staining, the gel on the slides was allowed to dry at room temperature to form a thin adherent film of dried stained gel on the glass. The precipitin bands were photographed by using the stained gel as a negative over photographic paper.

Direct Precipitin and Agglutinin Tests

The method used was that of Martin and Durham (1943). Before the tests were performed, both sera and reconstituted lyophilized antigen solutions were centrifuged thoroughly to remove any particulate matter. Lyophilized and live antigens of the three species were dissolved or suspended in normal saline to give a final concentration of 3.2 mg protein per ml. Serial double dilutions of this stock solution were made to give a series of final antigen concentrations ranging from 1.600 mg protein per ml to 0.00078125 mg protein per ml after mixing with equal parts of the various serum dilutions. The serial double serum dilutions, prepared in the same way gave final concentrations ranging from 1:1 to 1:128.

The tests were performed by mixing 0.5 ml quantities of the various antigens with the various antisera. The mixtures were shaken a few times and examined for precipitation or agglutination after standing for two hours at room temperature or at 37°C.

Absorption Tests

Each of the anti-Trichomonas spp.-live-antigen sera and the anti-Trichomonas spp.-lyophilized-antigen sera, was absorbed with

its heterologous live and lyophilized antigens, respectively. In each case, 0.1 ml amounts of antigen of each heterologous species were added to 2 ml of antiserum until no further precipitate was detected. The precipitate was allowed to form for two hours at room temperature or $37^{\circ}C$, then at $4^{\circ}C$ overnight, between additions of the antigen. The supernatant was tested by micro-agar-gel diffusion method (Crowle, 1958) against the antiserum's respective specific antigen. Sera and lyophilized antigens were centrifuged thoroughly before use. Normal sera versus each antigen and, after removal of trichomonads, culture media versus each antiserum, were tested as controls.

III. RESULTS

Culture Media and Growth Rate Studies

From the growth rate studies performed, it was found that the three species exhibited highest multiplication in Feinberg's medium (Figure 1) and lowest multiplication in fluid thioglycollate medium (Figure 4). In C.P.L.M. medium Tr. foetus exhibited higher multiplication than T. gallinae and T. vaginalis (Figure 3). T. gallinae gave the highest counts in three of the media namely Feinberg's, Diamond's and fluid thioglycollate, when measured at the highest growth point - 20 hours after inoculation - of the three species (Figures 1, 2, and 4), while T. vaginalis exhibited the lowest counts in all of the four media (Figures 1 to 4).

Staining

Table 1 shows that the nucleus and the cell membrane were stained quite sharply with chlorazol Black E, Giemsa's stain, Wright's stain and Heidenhain's iron hematoxylin. The flagella and undulating membranes were stained best with Giemsa's and Wright's stains, but were faintly stained with Best's Carmine. The axostyle was most satisfactorily stained with Wright's stain while the costa and the parabasal body with Heidenhain's iron hematoxylin. Best's Carmine and Papanicolaou's stain did not stain the costa or the parabasal body.

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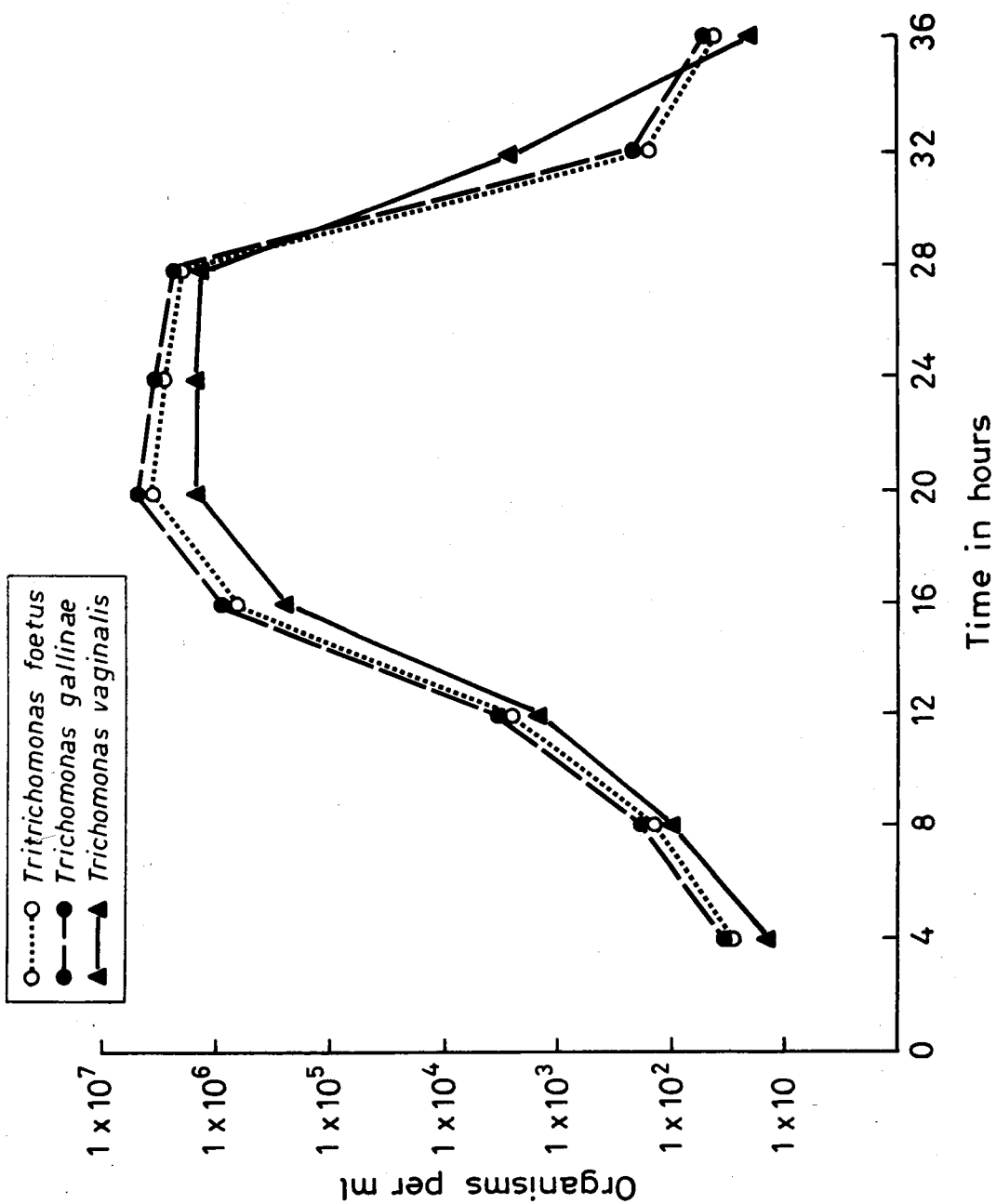
Figure 1. Growth rates of Tr. foetus, T. gallinae and T. vaginalis in Feinberg's Medium.
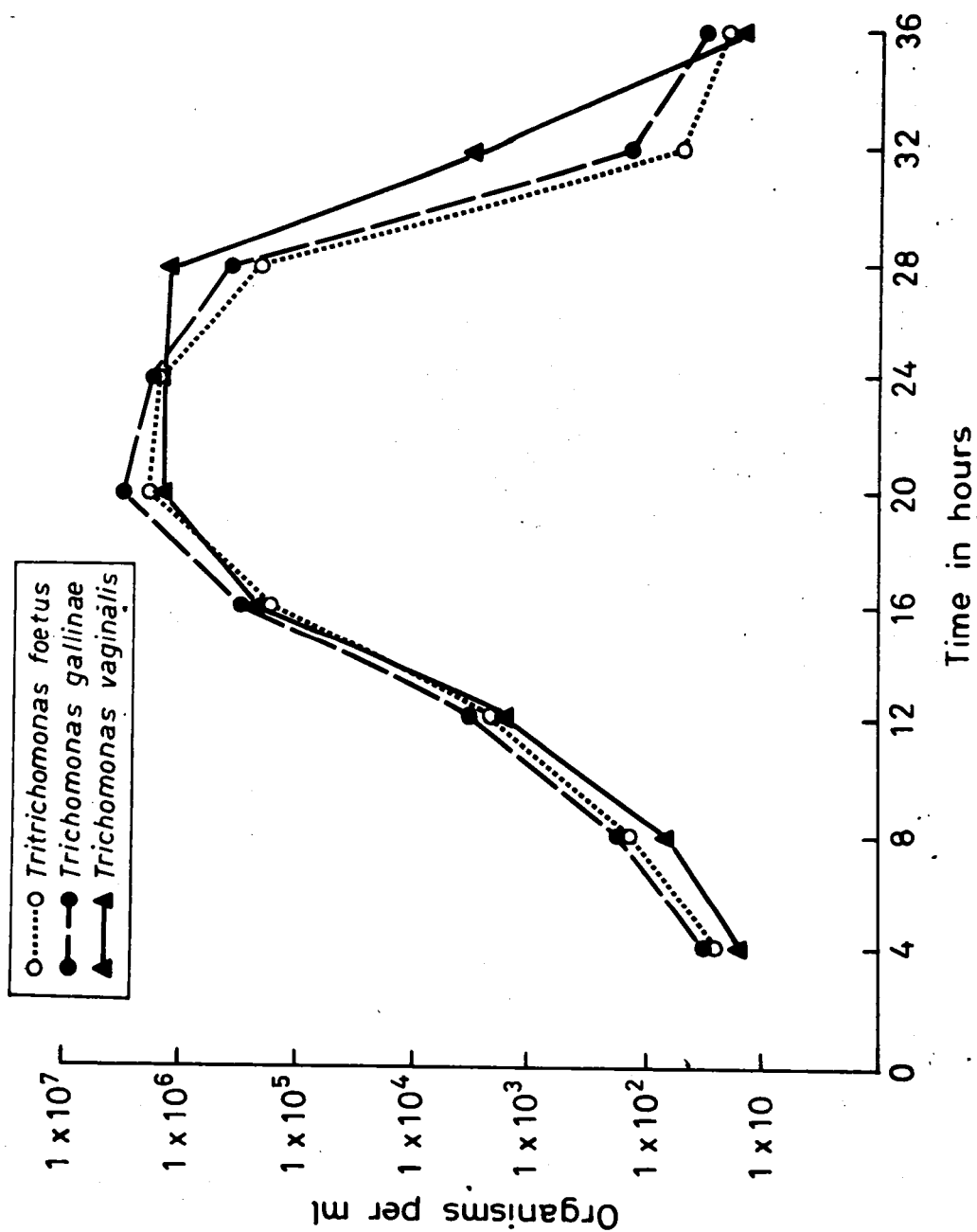
Figure 2. Growth rates of Tr. foetus, T. gallinae and T. vaginalis in Diamond's Medium
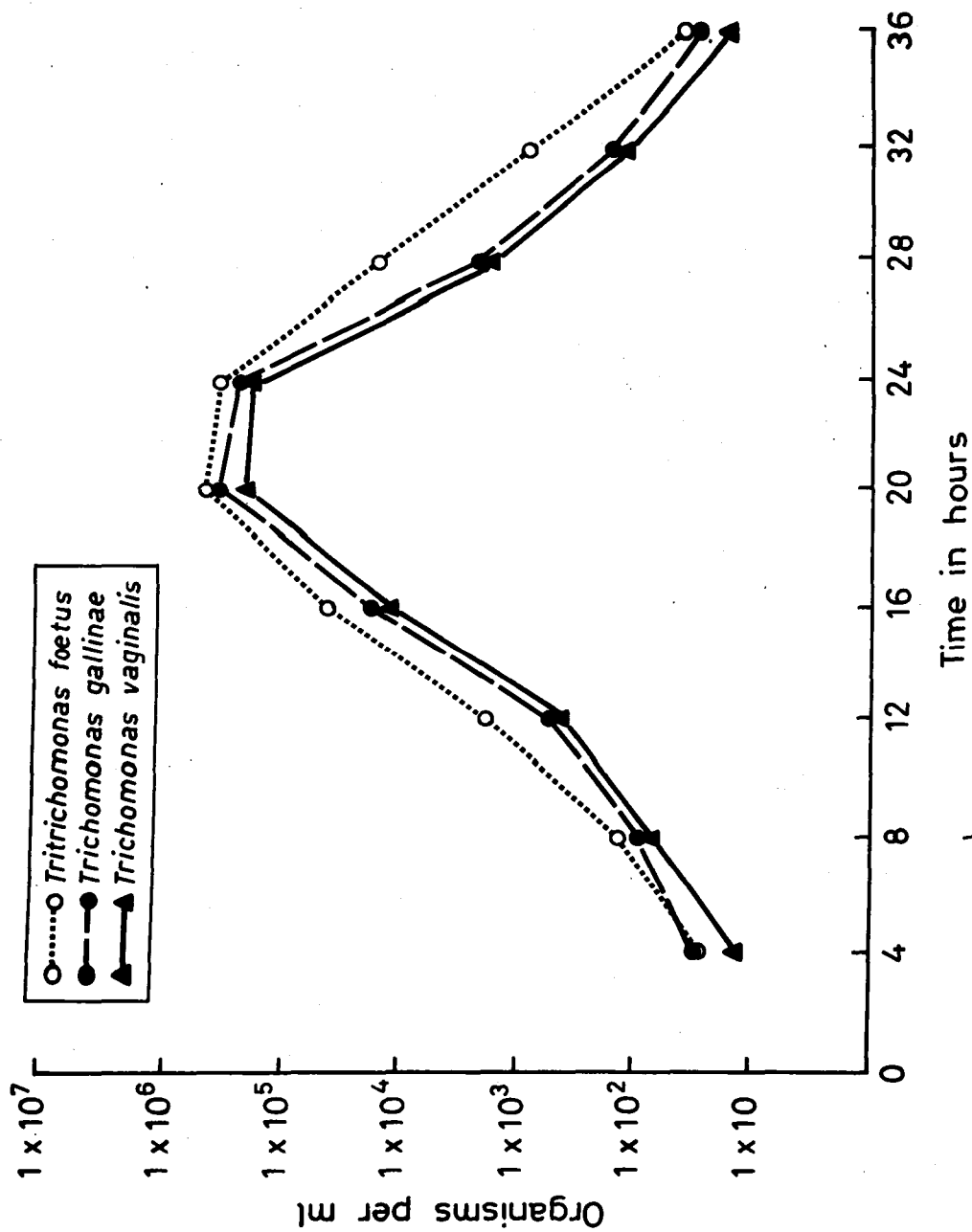
Figure 3. Growth rates of Tr. foetus, T. gallinae and T. vaginalis in C.P.L.M. Medium.
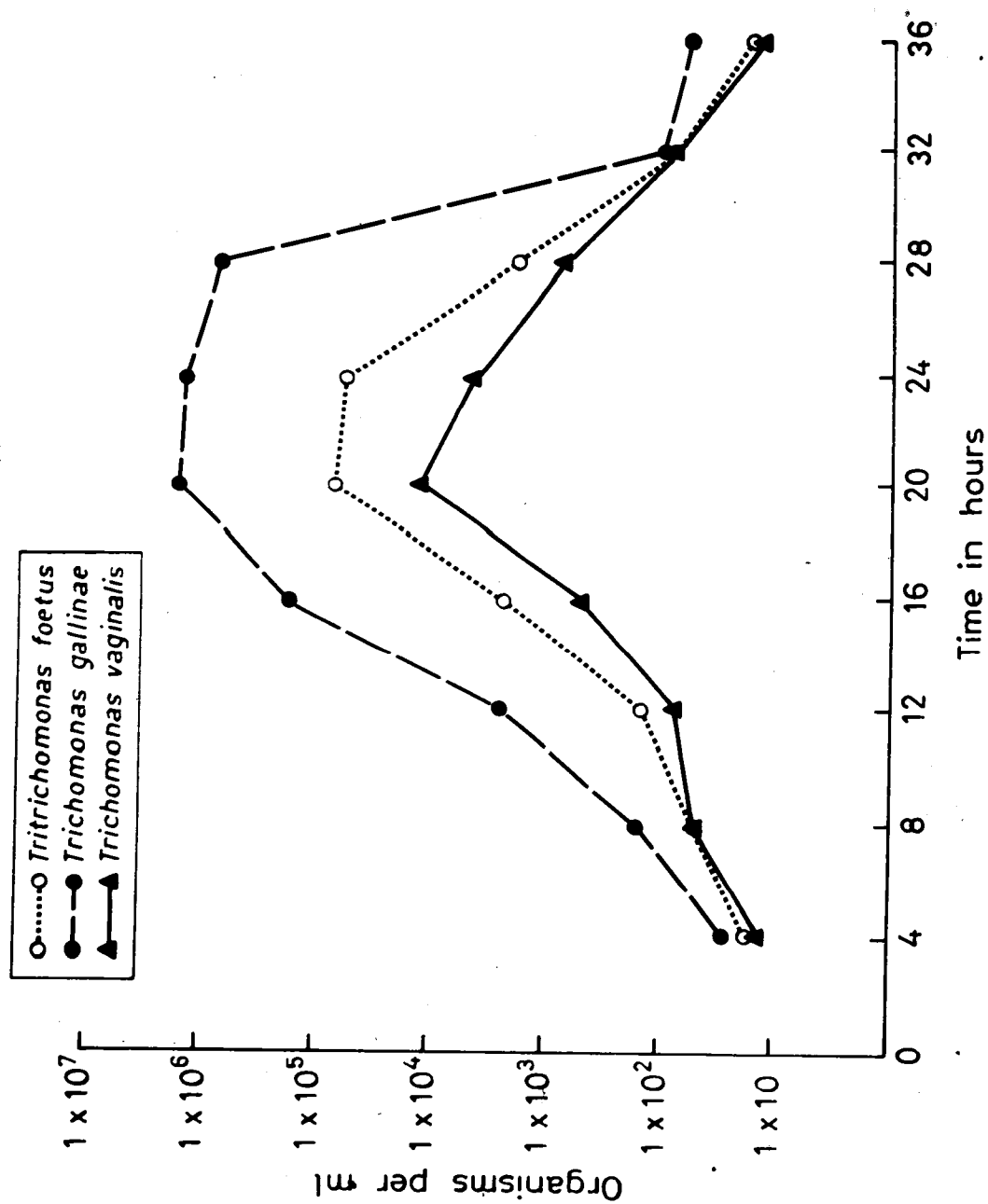
Figure 4. Growth rates of Tr. foetus, T. gallinae and T. vaginalis in Fluid Thioglycollate Medium.

Table 1. Selective demonstration of trichomonad organelles using six different staining techniques.

Stain	Cell membrane	Cytoplasm	Nucleus	Axostyle	Costa	Parabasal body	Flagella	Undulating membrane
Chlorazol Black E	++++	++	++++	+	±	±	+	+
Giemsa's	+++	+++	++	++	++	++	+++	++
Wright's	++	++	+++	++++	++	++	++++	+++
Heidenhain's Iron Hematoxylin	+++	++++	+++	++	+++	++++	++	++
Papanicolaou's	++	++	++	+	-	-	±	±
Best's Carmine	++	++	+	+	-	-	±	±

+ Positively stained
± Faintly stained
- Not stained

Micro-Agar-Gel Immunodiffusion Tests

Tr. foetus, T. gallinae and T. vaginalis antigens diffused against their respective rabbit anti-lyophilized-antigen sera to give eight to eleven lines (Figure 5), seven to nine lines (Figures 7 and 11) and four to six lines (Figure 9), while they diffused against their respective anti-live-antigen sera to give four to seven lines (Figure 6), three to four lines (Figure 8), and one to two lines (Figure 10). T. gallinae and Tr. foetus diffused against anti- T. gallinae- lyophilized-antigen serum (TgDAs) to give three lines of identity (Figure 11), while T. gallinae and T. vaginalis diffused against the same antibody to give six lines of identity (Figure 7). Reference to Figure 5 shows that Tr. foetus and T. vaginalis diffused against anti-Tr. foetus-lyophilized-antigen serum (TfDAs) to give five lines of identity while Tr. foetus and T. gallinae gave two lines of identity. Finally, when T. vaginalis and Tr. foetus diffused against anti-T. vaginalis-lyophilized-antigen serum (TvDAs), five lines of identity formed whereas when T. vaginalis and T. gallinae were tested against TvDAs, three lines of identity formed (Figure 9).

Feinberg's culture media, from which the trichomonads had been separated were tested in duplicate against the various rabbit antisera in order to determine whether the antigen had contained any absorbed media; no precipitin lines were detected. On the other hand when these media were injected, as antigens, into rabbits and antisera produced were separated and tested against these antigens

Figure 5.   Micro-agar-gel immunodiffusion of
the various trichomonad antigens
(peripheral wells) versus TfDAs
(central well).

Figure 6.   Micro-agar-gel immunodiffusion of
the various trichomonad antigens
(peripheral wells) versus TfLAs
(central well).
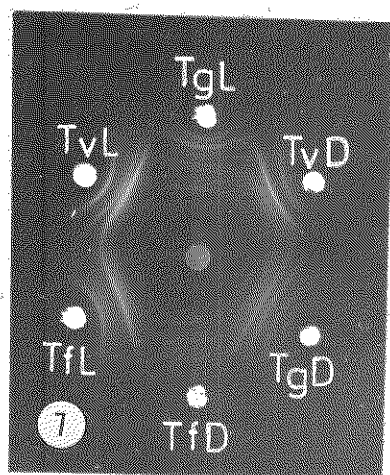
Figure 7.   Micro-agar-gel immunodiffusion of
            the various trichomonad antigens
            (peripheral wells) versus TgDAs
            (central well).

Figure 8.   Micro-agar-gel immunodiffusion of
            the various trichomonad antigens
            (peripheral wells) versus TgLAs
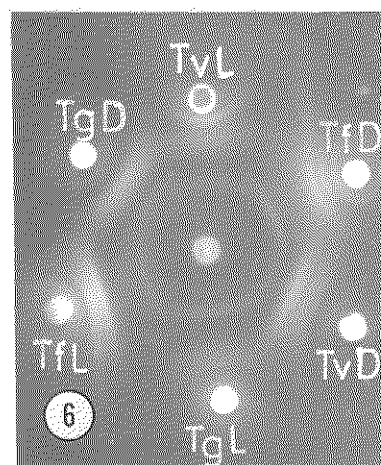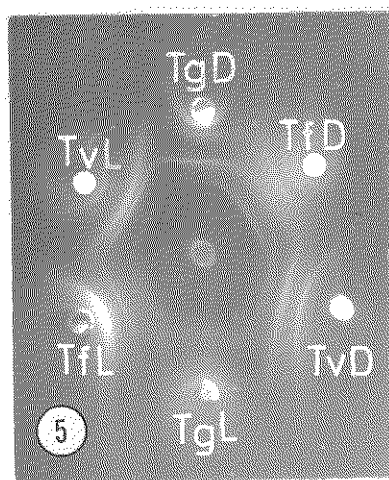   (central well).
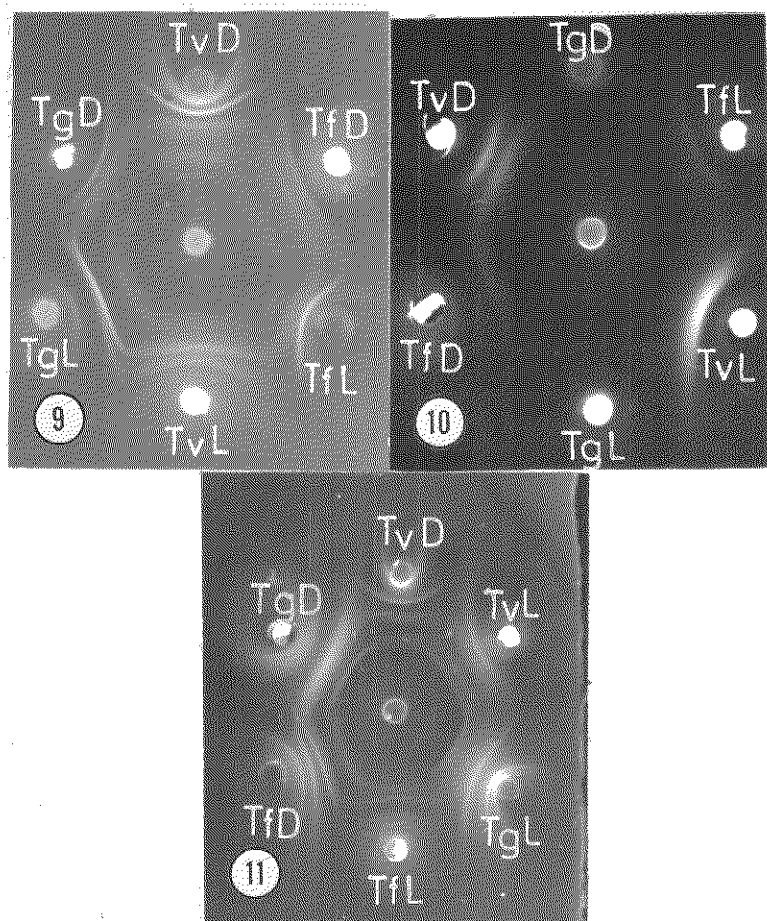
Figure 9. Micro-agar-gel immunodiffusion of the various trichomonad antigens (peripheral wells) versus TvDAs (central well).

Figure 10. Micro-agar-gel immunodiffusion of the various trichomonad antigens (peripheral wells) versus TvLAs (central well).
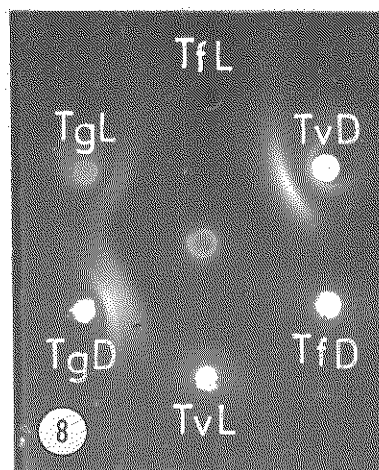
Figure 11. Micro-agar-gel immunodiffusion of the various trichomonad antigens (peripheral wells) versus TgDAs (central well).

and the lyophilized antigens of the three trichomonads, precipitin bands were observed very distinctly (Figures 12, 13, and 14).

Each of the antigen preparations was tested in duplicate against the rabbit sera collected before the immunization schedule. No precipitin bands formed in the gels.

The sera collected from the patients with trichomonad vaginitis and from pigeons infected with T. gallinae and also control sera collected from non-infected patients and pigeons, showed no precipitin band formation when tested against the different antigen preparations.

The precipitin bands stained markedly for proteins but were negative to the stains for polysaccharides and lipids.

Direct Precipitin and Agglutinin Tests

The results of these tests, exemplified in Tables 2 and 3, are recorded as + (precipitate or agglutinate) or - (no precipitate or no agglutinate); no differentiation being made as to the degree of flocculation or turbidity.

Antibody titration values (Table 4) were calculated as suggested by Marrack and Smith (1931) who took the unit of antibodies to represent the amount of antibodies equivalent to one microgram of antigen. The values are expressed in units per ml of undiluted serum.

Reference to Table 4, shows that TfDAs gave antibody titration values of 400 units per ml of undiluted serum when titrated against Tr. foetus, while TgDAs and TvBAs, gave values of 200 and 100 units per ml of undiluted serum, against T. gallinae and T. vaginalis,

Figure 12. Micro-agar-gel immunodiffusion of the
three trichomonad "exo-antigens",
TgX, TfX, and TvX, and TfD (peripheral
wells) versus anti-Tr. foetus -
"exo-antigen" serum (central well).
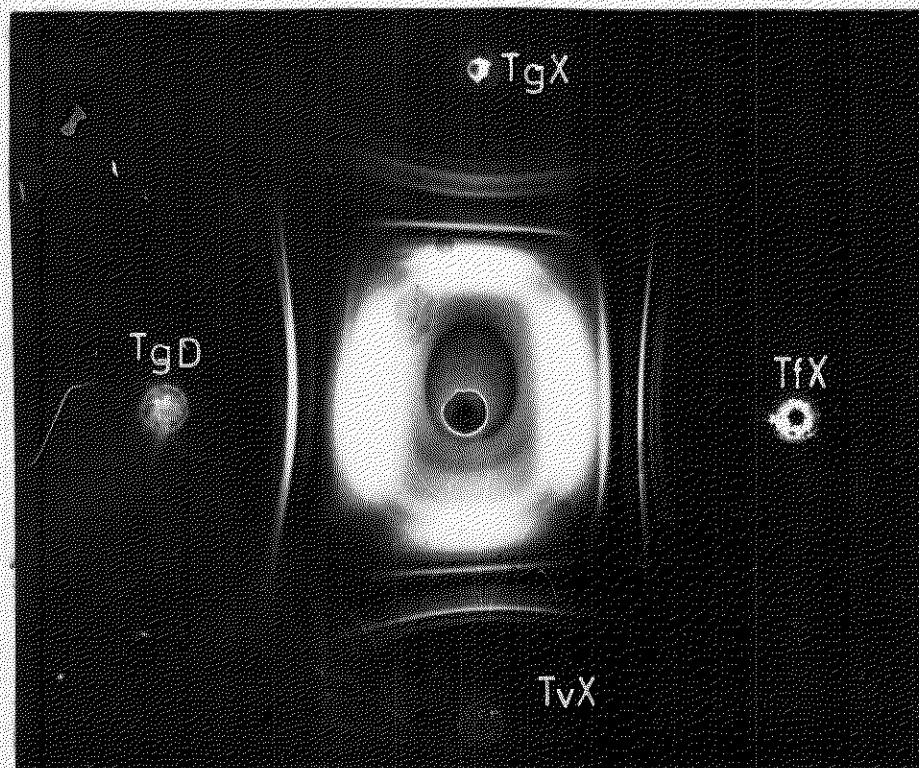
Figure 13. Micro-agar-gel immunodiffusion of the three trichomonad "exo-antigens", TgX, TfX and TvX, and TgD (peripheral wells) versus anti-T. gallinae-"exo-antigen" serum (central well).
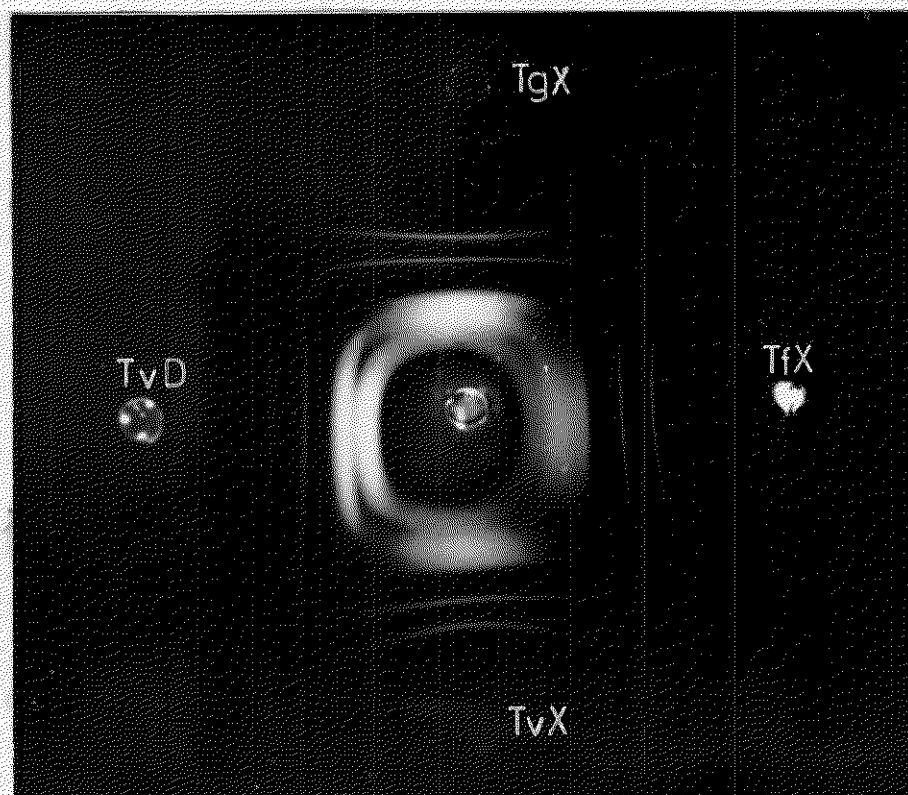
Figure 14. Micro-agar-gel immunodiffusion of the
three trichomonad "exo-antigens", TgX,
TfX and TvX, and TvD (peripheral wells)
versus anti-T. vaginalis-"exo-antigen"
serum (central well).

Table 2. Serial dilutions of TfDAs titrated against serial dilutions of TfD.

TfD expressed in micrograms protein per ml

Dilution of TfDAs	1,600	800	400	200	100	50	25	12.5	6.25	3.125	1.5625	0.78125
1:1	+	+	+	+	+	+	+	+	+	+	−	−
1:2	+	+	+	+	+	+	+	+	+	+	−	−
1:4	+	+	+	+	+	+	+	+	+	+	−	−
1:8	+	+	+	+	+	+	+	+	+	+	−	−
1:16	−	−	−	+	+	+	+	+	+	+	−	−
1:32	−	−	−	−	−	−	+	+	+	+	−	−
1:64	−	−	−	−	−	−	−	−	+	+	−	−
1:128	−	−	−	−	−	−	−	−	−	+	−	−

+   Precipitate
−   No precipitate
TfD   <u>Tr. foetus</u> lyophilized antigen
TfDAs   Anti-<u>Tr. foetus</u>-lyophilized-antigen sera

Table 3. Serial dilutions of TfDAs titrated against serial dilutions of TfL

TfL expressed in micrograms protein per ml

Dilution of TfDAs	1,600	800	400	200	100	50	25	12.5	6.25	3.125	1.5625	0.78125
1:1	+	+	+	+	+	+	+	+	+	–	–	–
1:2	+	+	+	+	+	+	+	+	+	–	–	–
1:4	+	+	+	+	+	+	+	+	+	–	–	–
1:8	–	–	–	–	–	+	+	+	+	–	–	–
1:16	–	–	–	–	–	–	–	+	+	–	–	–
1:32	–	–	–	–	–	–	–	+	+	–	–	–
1:64	–	–	–	–	–	–	–	–	–	–	–	–
1:128	–	–	–	–	–	–	–	–	–	–	–	–

+   Agglutinate
–   No agglutinate
TfL   Tr. foetus live antigen
TfDAs   Anti-Tr. Foetus-lyophillized - antigen sera

Table 4. Antibody titration values resulting from the
direct precipitin and agglutinin tests performed
between the various antisera and the trichomonads.

Antisera	TfD			TfL			TgD		
	Endpoints		Antibody titration value	Endpoints		Antibody titration value	Endpoints		Antibody titration value
	Ag	Ab		Ag	Ab		Ag	Ab	
TfDAs	3.125	1:128	400	6.25	1:64	400	3.125	1:8	25
TfLAs	3.125	1:32	100	6.25	1:16	100	3.125	1:4	12.5
TgDAs	3.125	1:8	25	6.25	1:4	25	3.125	1:64	200
TgLAs	-	-	-	-	-	-	3.125	1:16	50
TvDAs	6.25	1:16	100	12.5	1:8	100	6.25	1:4	25
TvLAs	3.125	1:4	12.5	3.125	1:4	12.5	-	-	-

Table 4 (Continued).

Antisera	TgL Endpoints Ag	TgL Endpoints Ab	TgL Antibody titration value	TvD Endpoints Ag	TvD Endpoints Ab	TvD Antibody titration value	TvL Endpoints Ag	TvL Endpoints Ab	TvL Antibody titration value
TfDAs	6.25	1:4	25	6.25	1:16	100	12.5	1:8	100
TfLAs	6.25	1:2	12.5	6.25	1:8	50	12.5	1:4	50
TgDAs	6.25	1:32	200	6.25	1:16	100	12.5	1:8	100
TgLAs	6.25	1:8	50	1.5625	1:4	6.25	3.125	1:2	6.25
TvDAs	12.5	1:2	25	3.125	1:32	100	6.25	1:16	100
TvLAs	-	-	-	3.125	1:8	25	6.25	1:4	25

x. Expressed in units of antibodies per ml of undiluted serum; the unit of antibodies being the amount of antibodies equivalent to 1 mg of antigen (Marrack and Smith, 1931).

TfD = Tr. foetus lyophilized antigen
TvD = T. vaginalis lyophilized antigen
TgD = T. gallinae lyophilized antigen
TfL = Tr. foetus live antigen
TvL = T. vaginalis live antigen
TgL = T. gallinae live antigen

TfDAs = Anti-Tr. Foetus-lyophilized-antigen serum
TvDAs = Anti-T. vaginalis-lyophilized-antigen serum
TgDAs = Anti-T. gallinae-lyophilized-antigen serum
TfLAs = Anti-Tr. Foetus-live-antigen serum
TvLAs = Anti-T. vaginalis-live-antigen serum
TgLAs = Anti-T. gallinae-live-antigen serum
Ag = Antigen
Ab = Antibody

respectively. However, anti-Tr. foetus-live-antigen serum (TfLAs), anti-T. gallinae-live-antigen serum (TgLAs) and anti-T. vaginalis-live-antigen serum (TvLAs), gave antibody titration values of 100, 50, and 25 units per ml of undiluted serum, when titrated against Tr. foetus, T. gallinae and T. vaginalis, respectively. Antibody titration values of 100 units per ml of undiluted serum resulted when TgDAs and TfDAs were titrated against T. vaginalis and when TvDAs was titrated against Tr. foetus; while values of 25 units per ml of undiluted serum were obtained when TfDAs and TvDAs were titrated against T. gallinae and when TgDAs was titrated against Tr. foetus.

No precipitation or agglutination occurred in the control tests run by the titration of Feinberg's culture media from which the trichomonads had been separated and the rabbits' sera collected before challenging the latter with the various antigens, against live and lyophilized antigens of the three species. Also, no precipitation or agglutination was detected when sera from infected patients and pigeons were titrated against live and lyophilized antigens of Tr. foetus, T. gallinae and T. vaginalis.

Absorption Tests

After absorption with T. vaginalis and Tr. foetus live antigens (TvL and TfL), TgLAs diffused against T. gallinae live antigen (TgL) to give one precipitin band (Figure 15). A similar precipitin band (Figure 16) was formed when TgDAs was tested against T. gallinae lyophilized antigen (TgD) after its absorption with T. vaginalis and Tr. foetus lyophilized antigens (TvD and TfD). No precipitin bands
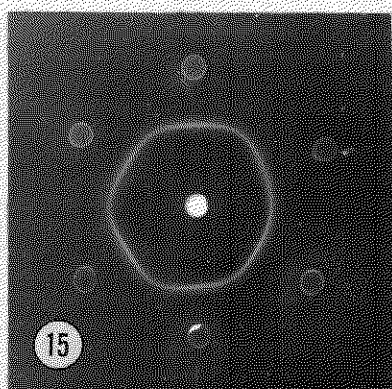
Figure 15. Micro-agar-gel immunodiffusion of
            absorbed TgLAs (central well)
            versus TgL (peripheral wells).
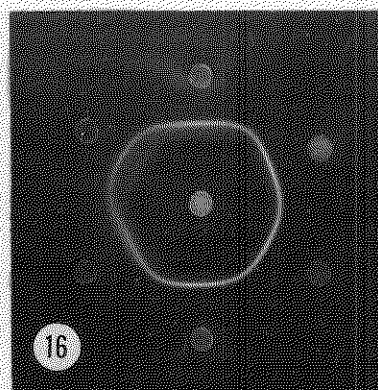

Figure 16. Micro-agar-gel immunodiffusion of
            absorbed TgDAs (central well)
            versus TgD (peripheral wells).

were formed when TvLAs or TvDAs were tested against TvL or TvD
after absorption with TgL or TgD and with TfL or TfD, nor were any
precipitin bands formed when TfLAs or TfDAs were tested against
TfL or TfD after absorption with TgL or TgD and TvL or TvD.

IV. DISCUSSION

It is obvious from the growth rate studies performed on the trichomonads, that of the four media used, namely Feinberg's medium, Diamond's medium, C.P.L.M. medium, and fluid thioglycollate medium: Feinberg's medium, followed by Diamond's medium, proved to be most satisfactory. In both of these media T. gallinae gave the highest counts and T. vaginalis the lowest. Optimum growth was reached in each of the artificial media at 20 hours after inoculation. These findings support McEntegart's work (McEntegart, 1954); he studied the growth of T. vaginalis, T. foetus (Belfast) and T. gallinae without bacteria, and T. hominis with a mixed bacterial flora. All bacteria-free cultures were grown in a slight modification of a medium described by Feinberg (Feinberg, 1953). McEntegart found that the strains growing without bacteria reached their peak of growth about 18 to 20 hours after inoculation and that at the height of growth, T. gallinae gave the highest counts and T. vaginalis the lowest.

Since the trichomonads showed a precipitous drop of growth 28 hours after inoculation into artificial media and a steep rise of growth at 16 hours after inoculation into clean media, it seemed most practical to subculture and harvest the organisms between 16 and 28 hours after inoculation; 20 hours being the optimal time.

Six staining methods for the selective demonstration of the various organelles of the trichomonads and consequently for the rapid

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identification of species were tried. It was found that Chlorazol Black E, Giemsa's stain, Wright's stain and Heidenhain's iron hematoxylin, were the best to sharply demonstrate the cell membrane and the nucleus. Giemsa's and Wright's stains were best for staining the flagella and the undulating membrane. The axostyle was stained best with Wright's stain while the costa and the parabasal body, with Heidenhain's iron hematoxylin. The costa and the parabasal body were not stained by Best's Carmine and Papanicolaou's staining method.

The present results indicate that for the accurate identification of the trichomonads as a whole, Heidenhain's iron hematoxylin and Wright's stain are the best. These are followed by Giemsa's stain. Chlorazol Black E may be used selectively to demonstrate accurately the cell membrane and the nucleus, while Papanicolaou's stain and Best's Carmine are of use only for demonstrating the presence of the parasite.

Trichomonads may also be compared by serological techniques. Since they are often endoparasites it is of interest to study their relative abilities to act as antigens with consequent production of specific antibody. Many workers in the field of parasitology and immunology have tried to evaluate different antigenic forms of the parasite. Trussell (1946) demonstrated that high titer antisera are produced when live organisms (T. vaginalis) were injected into rabbits. In differentiating between live and lyophilized antigens, Samuels and Chun-hoon (1964) found that a higher antibody titer was stimulated by live organisms (Tritrichomonas augusta) than by lyophilized

organisms. Reference to the serological results obtained in this study of the three trichomonads, namely Tr. foetus, T. gallinae and T. vaginalis, it was found that antisera to the lyophilized organisms gave a greater number of precipitin lines than antisera to the live organisms when tested against their respective antigens. There was also evidence that this form of the antigen stimulated a higher antibody response; this fact was supported by the direct precipitin and agglutinin tests, which showed that antibody content of antisera to lyophilized antigens was greater than that to anti-live-antigen sera. From the number of precipitin lines and antibody titration values, Tr. foetus was shown to produce higher antibody than T. gallinae which in its turn produced more than T. vaginalis. This indicates that Tr. foetus is a stronger antigen than T. gallinae which is a stronger antigen than T. vaginalis, under the conditions applying in these experiments.

Micro-agar-gel immunodiffusion tests showed that T. gallinae and T. vaginalis had six antigens in common while T. gallinae and Tr. foetus had three; this when T. gallinae, T. vaginalis, and Tr. foetus were tested against TgDAs. Tr. foetus and T. vaginalis had five antigens in common while Tr. foetus and T. gallinae had two when tested against TfDAs. However, T. vaginalis and Tr. foetus had five antigens in common while T. vaginalis and T. gallinae had three when tested against TvDAs. These results show, other than the existence of antigenic similarities between the species that TgDAs contains more antibodies to T. vaginalis than to Tr. foetus, that TfDAs contains more antibodies to T. vaginalis than to T. gallinae and that TvDAs

contains more antibodies to Tr. foetus than to T. gallinae. These implications are supported by the direct precipitin and agglutinin tests which gave antibody titration values of 100 units per ml of undiluted serum when TgDAs and TfDAs were titrated against T. vaginalis and when TvDAs was titrated against Tr. foetus; and 25 units per ml of undiluted serum when TfDAs and TvDAs were titrated against T. gallinae and when TgDAs was titrated against Tr. foetus.

Anti-T. gallinae serum after absorption with T. vaginalis and Tr. foetus formed one precipitin band against its homologous antigen, while no precipitin bands were formed after absorption of anti-Tr. foetus serum with T. vaginalis and T. gallinae or after absorption of anti-T. vaginalis serum with Tr. foetus and T. gallinae. This indicates that T. gallinae has one specific antigen apart from the antigens it has in common with T. vaginalis and Tr. foetus. This will lead to the belief in the presence of a "common antigenic pool" for the three trichomonads; this "pool" is most probably generic in nature. In this case T. gallinae apart from contributing most of its antigens to the "pool" has at least one specific antigenic component which it does not share with T. vaginalis and Tr. foetus.

Negative results obtained when Feinberg's culture media, from which the trichomonads had been separated, were tested against the various rabbit antisera, indicate that no adsorbed media were present when the three trichomonads were injected into rabbits. When these used media preparations were injected into rabbits, however, they produced antibody to the used media and to the trichomonad antigens. The precipitin bands in this system were particularly sharp. This

observation indicates that the media after removal of trichomonads contained a soluble-or exo-antigen from the trichomonads in small quantities, but sufficient to successfully immunize rabbits. This phenomenon was not studied further because it involved more time than could be spared.

Rabbit sera collected before the immunization schedule and tested against the different antigen preparations induced no precipitin band formation; these sera exhibited no precipitation or agglutination. This indicates the absence of natural antibodies, agglutinins or precipitins, against the three trichomonads in rabbits. Natural active antibody production, agglutinins or precipitins, were not demonstrated in sera collected from three patients with trichomonad vaginitis and three pigeons with T. gallinae infection. Also, no natural passive antibody production was demonstrated in the sera of three non-infected women and pigeons. These results are not statistically significant since the samples tested were neither adequate nor randomly chosen to represent a population.

The precipitin bands in all of the micro-agar-gel immuno-diffusion tests performed are predominantly proteinaceous in nature since they stained for proteins but not for polysaccharides and lipids.

In the light of the above serological tests it could be concluded that Tr. foetus, T. gallinae and T. vaginalis are antigenically related. Tr. foetus and T. vaginalis are almost serologically indistinguishable while T. vaginalis and T. gallinae, and Tr. foetus and T. gallinae are serologically distinct.

LITERATURE CITED

Amies, C.R., and M. Garabedian. 1965. Preparation and use of
fixative slides for the diagnosis of Trichomonas
vaginalis. J. Clin. Path. 18:27-30.

Anderson, E., and H.W. Beames. 1959. The cytology of Tritrichomonas
as revealed by electron microscopy. Jour. of Morphology.
104:205-236.

Asami, K., and M. Nakamura. 1955. Experimental inoculation of
bacteria-free Trichomonas vaginalis into human vaginae
and its effect on the glycogen content of vaginal
epithelia. Amer. Jour. Trop. Med. and Hyg. 4:254-258.

Asami, K., Y. Nodake, and T. Ueno. 1955. Cultivation of
Trichomonas vaginalis on solid medium. Exptl. Parasitol.
4:34-39.

Bauer, A.C., A. Epifanio, and W.J. Render. 1959. Cytology of
vaginal smears with Trichomonas. Obstet. and Gynecol.
14:381-388.

Björklund, B. 1954. Qualitative analysis of gel precipitates with
the aid of chemical color reactions. Proc. Soc. Exptl.
Biol. Med. 85:438-441.

Chakraborty, J., N.N. Das Gupta, and N.H. Ray. 1961. An electron
microscope study of Trichomonas criceti. Cytologia.
26:320-326.

Ciuca, T., and V. Mihalache. 1961. Differentiation between dead
and living parasites in urogenital trichomoniasis by
means of a 1:1,000 methylene blue solution.
Rumanian Med. Rev. 5:81-83.

Crowle, A.J. 1956. A simplified agar electrophoretic method for
use in antigen separation and serologic analysis. J. Lab.
Clin. Med. 48:642-648.

Crowle, A.J. 1958. A simplified micro double-diffusion agar
precipitin technique. J. Lab. Clin. Med. 52:784-787.

Crowle, A.J. 1961. Immunodiffusion. Academic Press, New York.

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

Feinberg, J.G. 1953. A method for the bulk growth of a parasitic protozoan. Nature. 171:1165.

Feinberg, J.G., and M.J. Whittington. 1957. A culture medium for Trichomonas vaginalis Donné and species of Candida. J. Clin. Path. 10:327-329.

Filadoro, F., and N. Orsi. 1958. Cultivation of Trichomonas vaginalis on solid medium and its application to the assay of Trichomycin potency. Antibiotics and Chemotherapy. 8:561-563.

Gleason, N.N., and G.R. Healy. 1965. Modification and evaluation of Kohn's one-step staining technic for intestinal protozoa in feces or tissue. Am. J. Clin. Path. 43:494-496.

Hayes, B.S., and E. Kotcher. 1960. Evaluation of techniques for the demonstration of Trichomonas vaginalis. Jour. Parasitol. 46 (5 Sect.2):45.

Hayward, B.J., and R. Augustin. 1957. Quantitative gel diffusion methods for assay of antigens and antibodies. Intern. Arch. Allergy Appl. Immunol. 11:192-205.

Heremans, J., and J.P. Vaerman. 1958. Différenciation sur papier des protéines et des mucopolysaccharides acides au moyen de colarations spécifiques combinées. Clin. Chim. Acta. 3:430-434.

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

Honigberg, B.M., and H.A. Davenport. 1954. Staining of flagellate protozoa by various silver-protein compounds. Stain Technol. 29:241-246.

Honigberg, B.M., V.K. Farris, and M.C. Livingston. 1965. Preservation of Trichomonas vaginalis and Trichomonas gallinae in liquid nitrogen. In: Progress in Protozoology. (Abstr. II. Int'l. Conf. Protozool., London). Excerpta Medica Foundation. Amsterdam, New York, London, Milan, Tokyo, Buenos Aires. pp 199.

Honigberg, B.M., and V.M. King. Structure of <u>Trichomonas vaginalis</u>
Donné. Jour. Parasitol. 50:345-364

Inoki, S., K. Nakanishi, and T. Nakabayashi. 1960. Electron
microscopic observations on <u>Trichomonas vaginalis</u>
employing the thin-section technique. Gynecologia.
149 (Suppl.):48-54.

Ivey, M.H. 1961. Growth characteristics of clones of <u>Trichomonas</u>
in solid medium. Jour. Parasitol. 47:539-544.

Jensen, E.A., and D.M. Hammond. 1964. A morphological study
of trichomonads and related flagellates from the
bovine digestive tract. Jour. Protozool. 11:386-394.

Johnson, A.E. Comparison of six strains of <u>Trichomonas foetus</u>.
II. Gel-diffusion precipitin studies of soluble
antigens. Exptl. Parasitol. 21:336-343.

Johnson G., and R.E. Trussell. 1943. Experimental basis for
the chemotherapy of <u>Trichomonas vaginalis</u>
infestations. Proc. Soc. Exptl. Biol. Med.
54:245-249.

Joyner, L.P., and G.H. Bennett. 1956. Observations on the
viability of <u>Trichomonas foetus</u> during the process
of freezing to -79°C and thawing in the presence
of glycerol. J. Hyg. 54:335-341.

Kabat, E.A., and M.M. Mayer. 1964. <u>Experimental Immunochemistry</u>.
Charles C. Thomas, Springfield, Ill. 2nd ed.

Kohn, J. 1957. An immuno-electrophoretic technique. Nature.
180:986-987.

Kohn, J. 1958. Small-scale membrane filter electrophoresis and
immuno-electrophoresis. Clin. Chim. Acta.
3:450-454.

Kudo, R.R. 1954. <u>Protozoology</u>. Charles C. Thomas, Springfield,
Ill., 4th ed.

Kupferberg, A.B., G. Johnson, and H. Sprince. 1948. Nutritional
requirement of <u>Trichomonas vaginalis</u>. Proc. Soc. Exp.
Biol. Med. 67:304-308.

Lash, J.J. 1950. A simplified casein hydrolysate-serum medium
for the cultivation of <u>Trichomonas vaginalis</u>. Amer.
Jour. Trop. Med. 30:641-642.

Levine, N.D. 1961. Protozoan Parasites of Domestic Animals and of Man. Burgess Publishing Co., Minneapolis, Minnesota.

Levine, N.D., and W.C. Marquardt. 1954. The effect of glycerol on survival of Tritrichomonas foetus at freezing temperatures. Jour. Protozool. 1(Suppl.):4.

Lowe, G.H. 1965. A comparison of current laboratory methods of a new semi-solid culture medium for the detection of Trichomonas vaginalis. J. Clin. Path. 18:432-434.

Lynch, K.M. 1915. Trichomoniasis of the vagina and mouth. Am. J. Trop. Disease Prev. Med. 2:627.

Macdonald, E.A., and A.L. Tatum. 1948. The differentiation of species of trichomonads by immunological methods. Jour. Immunol. 59:309.

Markham, R. 1942. A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem. J. 36:790-791.

Mandal, L.N., H. Prasad, and A.K. Prasad. 1963. Observations on the staining techniques of Trichomonas foetus. Indian Veterinary J. 40:263-265.

Marrack, J., and F.C. Smith. 1931. Quantitative aspects of immunity reactions: The precipitin reaction. British J. Exper. Path. 12:30.

Martin, D.S., and N.C. Durham. 1943. A simplified serum dilution method for the quantitative titration of precipitins in a pure antigen-antibody system. J. Lab. Clin. Med. 28:1477-1482.

McEntegart, M.G. 1954. The maintenance of stock strains of trichomonads by freezing. J. Hyg. 52:545-550.

McEntegart, M.G. 1956. Serological comparison of a strain of Trichomonas vaginalis with the Belfast and Manley strains of Trichomonas foetus. Jour. Path. and Bact. 71:111-115.

Menolasino, N.J., and E. Hartman. 1954. Immunology and serology of some parasitic protozoan flagellates. I. Trichomonas vaginalis and Trichomonas foetus. Jour. Immunol. 72:172-176.

Papanicolaou, G.N. 1954. Atlas of Exfoliative Cytology. Harvard University Press, Cambridge.

Robertson, M. 1960. The antigens of Trichomonas foetus isolated from cows and pigs. J. Hyg. 58:207-213.

Samuels, R. 1965. Growth of axenic trichomonads in a serum-free medium. In: Progress in Protozoology. (Abstr. II. Int'l. Conf. Protozool., London). Excerpta Medica Foundation. Amsterdam, New York, London, Milan, Tokyo, Buenos Aires. pp. 200.

Samuels, R., and H. Chun-hoon. 1964. Serological investigations of trichomonads. I. Comparisons of "natural" and immune antibodies. Jour. Protozool. 11:36-46.

Samuels, R., and D.J. Steuder. 1960. Experiments with trichomonads grown as colonies in agar plates. Jour. Protozool. 7(Suppl.):6.

Sanborn, W.R. 1955. Microagglutination reactions of Trichomonas suis, T. sp., and T. foetus. Jour. Parasitol. 41:295-298.

Shute, P.G. 1966. The staining of malaria parasites. Trans. Roy. Soc. Trop. Med. and Hyg. 60:412-416.

Simpson, C.F., and F.H. White. 1964. Structure of Trichomonas foetus as revealed by electron microscopy. Am. J. Vet. Res. 25:815-824.

Smith, B.F., and B.T. Stewart. 1966. Fine structure of Trichomonas vaginalis. Exptl. Parasitol. 19:52-63.

Trussell, R.E. 1946. Microagglutination tests with Trichomonas vaginalis. Jour. Parasitol. 32:563-569.

Uriel, J. 1958. Colorimetric detection of human caeruleplasmin oxidase activity after electrophoresis in agar plates or after immuno-electrophoresis. Nature. 181:999-1000.

Weinman, D., and J. McAllister. 1947. Prolonged storage of human pathogenic protozoa with conservation of virulence: observations on the storage of helminths and leptospiras. Am. J. Hyg. 45:102.

Wirtschafter, S.K. 1954. The maintenance of Trichomonas vaginalis on a solid medium. Jour. Parasitol. 40:100. 1954.

Zhuravskii, P.V. 1962. A method for staining Trichomonas. Lab. Delo. 8:58.