SOME ASPECTS OF THE CARBOHYDRATE METABOLISM OF CERTAIN TRICHOMONADS

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SOME ASPECTS OF THE CARBOHYDRATE METABOLISM OF CERTAIN TRICHOMONADS

By

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INTRODUCTION

Studies of the chemical activities of micro-organisms have been largely confined to the bacteria. The primary reason for this has been the relative ease with which large quantities of bacteria are cultured, compared to the difficulties encountered with other organisms, especially the protozoa. With the development of more satisfactory methods for growth of protozoa information on their metabolic activities has been gradually accumulating.

This information, however, is still very sketchy and incomplete. Only a few species have been studied and for these there is but little quantitative information. It has been found that protozoa do not always follow the well-travelled metabolic pathways found in the bacteria, but carry on their cellular chemistry with many modifications and innovations - the details of which are still obscure. Much of the work in this area of research to date has logically been concerned with accumulating basic information regarding the type of metabolism possible, the substrates utilized and those which are necessary, the optimum conditions for the organism's physiological activities, and the effects of various external agents on growth and metabolism. A few reports on characterization of intracellular enzymes and cell

contents have appeared but these are the exception.

It is obvious that the study of the biochemistry of protozoa lags far behind that of the bacteria. This is not only a consequence of the problems inherent in working with these organisms but is also due indirectly to the fact that these organisms have been most often studied by biologists who were not specially trained in chemistry. These workers concentrated on elucidating the often complex life cycles of these organisms, and describing their cellular morphology, but did not until recently consider their biochemistry.

These barriers are being rapidly removed by the entrance into basic biological research of men trained in chemistry as well as biology.

Still, a mass of basic information remains to be detailed. Many more protozoa must be grown in pure culture and investigated for possibly hitherto unknown growth factors, must be examined for their activities in protein, carbohydrate, and lipid metabolism, and information obtained regarding their enzyme content. The enzymes must be characterized and then compared with those found in other protozoa and other micro-organisms whose chemistry is better understood in order to make the greatest contribution to comparative biochemistry.

The flagellate, Trichomonas vaginalis, because of

its importance as a parasite of humans, is one of the organisms which has received some attention in this regard. Methods for growing the organism are available and some of its requirements are known. Other workers have produced brief reports on similar work with Tri-trichomonas foetus, a pathogen of cattle.

However, to date there has been no regular report of research on the metabolic activities of other species of Trichomonas or related forms with an attempt to discover differences, if any, and to relate these to parasitic habitat.

It was the purpose of this work to investigate the application of methods, which had been found to be of value in the study of other microbes, to the identification of some of the biochemical processes in the carbohydrate metabolism of four species of trichomonads.

REVIEW OF THE LITERATURE

A. The parasites

The organisms known variously as Trichomonas, Tritrichomonas, Pentatrichomonas, Trichomastix, etc., are flagellates of the order Trichomonadida Kirby, 1947. Those with which the present work is concerned are placed by Kirby (1947) in the family Trichomonadidae Wenyon, 1926. At least 90 species of these organisms have been described (Morgan, 1944), and until Kirby proposed a reclassification the taxonomy was rather confused. Even now, all workers evidently do not accept Kirby's terminology. For example, LaPage (1956), Ryley (1955a, 1955b), and Menalosino and Hartman (1954) prefer to place these organisms in the one genus Trichomonas. Kirby's classification is used in this work.

These protozoa are somewhat pyriform in shape, the posterior end more pointed than the anterior, with 3 to 5 anteriorly-attached flagella. One of these is directed backwards and united with the body by a well-developed undulating membrane. A stiff fibril, called the costa , is seen to run along the base of the undulating membrane. In some species a row of deeply staining chromatin granules is present near the costa. A supporting structure, giving stiffness to the body, and often projecting from the posterior end, is the axostyle. Granules are sometimes seen within this structure also. There is a

single, oval nucleus near the anterior end of the axostyle. A slit-like cytostome is located anteriorly on the side opposite the undulating membrane.

The organisms divide by longitudinal fission and, although there are some reports to the contrary, are not thought to have a cystic stage.

The organisms in the present study were Tritrichomonas foetus (Riedmüller, 1928) Wenrich and Emmerson, 1933, a parasite of the reproductive tract of cattle (a cause of abortion); Pentatrichomonas gallinarum (Martin and Robertson, 1912) Mesnil, 1914, a parasite of gallinaceous birds; and Trichomonas suis Gruby and Delefond, 1843, a commensal from the intestinal tract of the pig (Sus scrofa). Of the latter, two separate isolates were studied. One was from the normal intestinal habitat. The other was from the nasal passages (turbinates) of a pig with atrophic rhinitis. Although attempts have been made to incriminate this organism as the cause of the condition (Spindler, et al., 1953; Switzer, 1951), others (Levine, et al., 1954; Lindquist and Sanborn, 1953) have not been able to produce the infection under experimental conditions. It is not yet known what relation, if any, the two organisms have to each other.

Buttrey (1956) has reported that the so-called "fecal form" of T. suis is actually a mixture of two types of organisms, a large form and a small form, both

of which are distinctly different from that found in the nasal cavity. He believes there is morphologic similarity between the "nasal form" and T. foetus, and calls it a Tritrichomonas.

Sanborn (1955) and Rothenbacher (1956) found that serological differences existed when they produced antibodies to these organisms in rabbits.

For purposes of this report they will be referred to as the nasal and fecal forms of T. suis.

B. Protozoan physiology and metabolism

1. General - The biochemistry of protozoa was a rather neglected field of research until about 1951. Although some reports had appeared, they were generally unrelated. They concerned the basic nutritional needs of various parasitic flagellates, amoebae, and ciliates; with only a small amount of work on metabolism. A collection of the pertinent data to 1951 was published by Lwoff (1951). An elaboration of some of the metabolic work (primarily on flagellates), and suggestions for future research, was presented by von Brand (1952).

The most recent general review (Hutner and Lwoff, 1955) gives a resume of investigations beyond those in the 1951 volume. Discussions are included on comparative flagellate biochemistry (mostly free-living organisms), ciliate nutrition (especially the interesting steroid

requirements of Paramecium) and metabolism, slime mold development, mutualistic protozoa, and the physiological basis of chemotherapy of parasitic disease.

Despite these excellent recent advances, the amount of information regarding protozoan life processes seems very small when compared to other organisms. Many protozoa have not been grown in pure culture, let alone studied chemically. The basic metabolic patterns, so long familiar in other cells, are often extremely modified in protozoa; and details about these aberrant pathways are lacking. Also, growth factors necessary for some protozoa turn out to be related to metazoan vitamins and hormones. In general, a large mass of detail must first be accumulated before generalizations logically can be made. It would seem that two statements by Hutner (1955) are pertinent here. He says, when speaking of the vast amount of work on cultures which yet remains: "...it may demand courage - even the taking of vows of academic poverty - to pursue the lonely, risky enterprise of taming new organisms, yet the growth of protozoology owes more to these pioneers than to those who in biochemical language belabor the point that protozoal protoplasm resembles other protoplasm." Again, in reference to the use of fundamental research rather than direct, more immediately useful approaches, he says: "(There is) danger (in) the temptation to attack difficult but important problems uneconomically - by frontal

assault. The meager results of this strategy when applied to the . . . exacting parasitic protozoa (are obvious). Perhaps the lasting value of these volumes may be their hints on how such scientific outposts may be outflanked when they cannot be stormed."

2. Flagellates - The flagellates which have received the most attention from researchers are the hemo-parasitic Trypanosomidae, which have primitive relatives in the green algae, the chrysomonads (from brown algal stock), and Trichomonas (also from green stock, but farther removed than the trypanosomes).

Study of the carotenoid pigments of these primitive organisms indicates a sexual function for them (Hutner and Provasoli, 1955).

The pathogenic trypanosomes have been studied by several workers (e.g. von Brand, 1952; Harvey, 1949; Ryley, 1956). Most of them have been found to possess a conventional Meyerhof-Embden glycolytic pathway at least as far as pyruvate, and an oxidative system insensitive to cyanide.

Phagotropy has been studied in Paranema, a protozoan related to the green Euglena but without chlorophyll and therefore more 'animal' in nature. These experiments, designed to analyze, if possible, the biochemical meaning of "plant vs. animal", have shown a more specific lipid requirement for Paranema. Several flagellates

have been shown to require vitamin B12 - either intact or in a folic acid form (Hutner, 1955).

A group of organisms (e.g. Chilomonas) have been found to utilize acetate as a sole source of carbon. That these "acetate flagellates" have an extremely different intermediary metabolism is becoming apparent. Recent work has shown that sugar phosphates are somehow involved in their metabolism (Hutner and Provasoli, 1955).

A few organisms (e.g. Euglena, Chilomonas, Chlamydomonas) give evidence of a conventional tricarboxylic acid (Krebs) cycle (Hutner and Provasoli, 1955).

Intracellular polysaccharides have been isolated from several protozoa. One of these, paramylum (from Euglena), is a 1-3' glucan, a form unknown in plants, metazoa, or bacteria (Hutner and Provasoli, 1955). An intracellular starch has also been purified from Polytomella coeca (Barker and Bourne, 1955).

3. Trichomonas - Since the present work concerns trichomonad metabolism, the reports of previous research will be considered in greater detail.

Witte (1933) showed that T. foetus could tolerate a pH range of 5.5 to 8.5. Cailleau (1937a) and Riedmüller (1936) reported that pH 6.5-7.6 was the optimum range for T. foetus in culture. Morista (1939) found the optimum pH to be 6.6-7.8 with the limits 5.6 and 8.4 compatible with survival. Johnson (1940)and Trussell

and Johnson (1941) found T. vaginalis to reach maximum population densities in culture at a pH value between 5.5 and 6.0 The extreme ranges, beyond which multiplication ceased, were 5.0 and 7.55. Morgan (1942) also studied the pH of cultures of T. foetus and reported the final value (when all organisms were dead) was usually 5.3 to 5.5. He also found (Morgan and Whitehair, 1943) that this organism lives in the reproductive tract of cows at a pH of about 7.1-7.2. The only report at variance with these is that of Tanabe (1925) who reported the optimum pH for culture of trichomonads from man, rat, and owl to be 8.0-8.2 in a complex medium.

In 1941 Lyford published results she obtained by varying the environment of bacteria-free cultures of T. foetus. Some of her more pertinent results follow:

(a) the organism was found to require complex nutrients such as egg yolk, blood, or serum - the latter allowing best growth when used in 40% concentration;

(b) the maximum population depended on the number of organisms inoculated;

(c) growth of T. foetus in a medium containing dextrose resulted in a decrease in pH of the medium to about 4.8;

(d) addition of as little as 1% of old culture filtrates to new culture medium resulted in decreased maximum population density. The inhibiting substance

(presumably a metabolite) was not identified;

(e) the glycogen content of the cells (as determined by direct inspection of iodine-stained organisms) became progressively less as a culture aged.

There have appeared several articles dealing with the nutritional requirements of T. vaginalis (Sprince and Kupferberg,1947a, 1947b; Sprince, 1948; Kupferberg, et al., 1948; Sprince, et al., 1949). In general these have dealt with the unknown factor(s) in blood serum necessary for growth of the organism in bacteria-free culture. A requirement for pantothenic acid and phosphate has been established, but a completely defined medium has not been developed.

Specific metabolic reactions of the various trichomonads have received little attention until relatively recently. von Brand (1950) said "...it is singular that the easily available trichomonads have so far received but little attention...". Earlier work (Cailleau, 1937a) indicated that T. foetus utilized glucose, galactose, lactose, fructose, maltose, saccharose, raffinose, dextrin, and soluble starch with a fall in culture pH to 5.7 - 6.0, but glycerol was not fermented. Another species, T. columbae (= T. gallinae), used glucose, galactose, maltose, saccharose, dextrin, and soluble starch, but only weakly fermented fructose, lactose, and inulin. This same worker (Cailleau, 1937b) also

reported in some detail on utilization of sterols by trichomonads.

Later, Trussell and Johnson (1941) found that T. vaginalis fermented glucose, maltose, starch, dextrin, and glycogen in aerobic culture. Fructose and galactose were only slightly utilized, and lactose was not attacked.

Willems, Massart, and Peeters (1942) were the first to apply manometric techniques to the study of the metabolism of a trichomonad. Using T. hepatica (= T. gallinae) they found that glucose, fructose, and mannose were utilized with an average respiratory quotient of 0.87 in pH 7.4 phosphate buffer. (It is interesting to note that they used only 10^6 organisms in each respirometer flask which gave extremely small oxygen uptakes - of the order of 6 microliters per hour.) Lactate, pyruvate, succinate, hexose diphosphate, and glucose-1-phosphate were not oxidized. The oxygen uptake was unaffected by cyanide, azide, fluoride, or 2,4-dinitrophenol.

Suzuoki and Suzuoki (1951a) were the first to study carbohydrate metabolism in T. foetus to any great extent. They found that oxygen uptake with glucose as substrate was maximal at a sodium chloride concentration of 0.19-0.26 M and a pH of 7.0-7.6. Of 27 substrates tested, only glucose, fructose, mannose, galactose, sucrose, and maltose stimulated respiration appreciably; while C_4

dicarboxylic acids were neither utilized nor activating. Pyruvate and lactate were not oxidized aerobically.

Studies of inhibitors showed that these characteristically antagonistic to enzymes of the Meyerhof-Embden glycolytic pathway (iodoacetate and fluoride) were most effective in decreasing oxygen uptake when glucose was used as substrate. Iodoacetate inhibited respiration 50% at a concentration of about 2.5×10^{-5} M, and fluoride was equally as effective at about 0.001 M. Hydroxylamine and cyanide were ineffective at concentrations less than 0.01 M, and azide only inhibited respiration about 12% at a concentration of 0.1 M. 2,4-dinitrophenol produced about 75% inhibition at a concentration of approximately 0.01 M.

Evidence was presented that for every molecule of oxygen consumed during respiration, one acid equivalent was formed per molecule of glucose used. The major acid produced was found to be succinic acid. Small amounts of lactic and pyruvic acids were also identified.

These workers (Suzuoki and Suzuoki, 1951a, 1951b) also presented evidence that hydrogen was produced from glucose by T. foetus under anaerobic conditions, a fact earlier reported by Andrews and von Brand (1938). Pyruvate and formate stimulated the endogenous hydrogen production only slightly. Formic dehydrogenase was identified in the cells, but no evidence could be found

for either formic hydrogenlyase or hydrogenase.

The presence of catalase and cytochrome b, but not cytochromes a or c, was demonstrated.

In a later paper from Japan (Ninomiya and Suzuoki, 1952) the metabolism of T. vaginalis was discussed, and comparisons made with T. foetus. Optimum conditions for respiration in the presence of glucose were given as 0.1-0.2 M NaCl, pH 4.5-6.0, and 5% or less oxygen. Increase in oxygen tension resulted in a decreased rate of respiration for T. vaginalis (also shown by Johnson, 1942) but an increased rate for T. foetus. It was shown that T. vaginalis possessed no catalase activity as did T. foetus so it was postulated that accumulation of hydrogen peroxide accounted for the inhibitory effect of oxygen.

Of 13 substrates tested with T. vaginalis, maltose and glucose were oxidized rapidly, lactate and pyruvate were oxidized at a rate about half that for glucose, and the others (including formate and acetate) were not utilized at all. The oxygen uptake was inhibited by iodoacetate, arsenite, fluoride, and p-mercuribenzoate. Cyanide, malonate, and azide had no effect. The inhibition by sulfhydryl-binding agents was considered by Baernstein (1955) as presumptive evidence for the presence of triosephosphate dehydrogenase.

It was found that glucose increased hydrogen production to about four times the endogenous level, while

pyruvate increased it only about twice the endogenous. Hydrogen evolution with glucose as substrate was maximal in phosphate buffer at about pH 6.2.

Kupferberg, et al., (1953) followed cultures of T. vaginalis in simplified trypticase serum (STS) medium Kupferberg, et al., 1948). They noted a change in pH of the medium from 6.0 to 5.75 in 48 hours with an increase in population from about 10,000 per ml to 1,800,000 per ml. Lactic acid was found to be the major acid produced by this organism in this medium. Its production paralleled the pH change.

Their studies on oxygen uptake in the presence of glucose showed that respiration was of the order of 162 microliters per 10^8 cells per hour, that it was proportional to the number of cells employed, and that the respiratory quotient decreased with time of respiration.

A CO_2-fixation product was isolated using radioactive carbon dioxide, but its nature was not determined.

In conflict with the reports of Ninomiya and Suzuoki (1952) and Read (1953) no gas other than CO_2 was identified by these workers as a product of T. vaginalis metabolism. Read and Rothman (1955) reported later that this organism produces hydrogen only under anaerobic conditions.

By far the most detailed work on trichomonad metabolism was reported by Ryley (1955a). In his work on T. foetus attention was focused mainly on the glycogen

reserves stored by the flagellate. An earlier paper (Stewart, 1938) had reported that as growth progresses (and as carbohydrate is consumed) in culture, the number and per cent of forms containing glycogen inclusions (as identified with iodine stain) increases to the time of maximum population and decreases thereafter. Using 48-hour cultures Ryley obtained an average endogenous qO_2 (microliters O_2/mg N/hr) of 176.

Of 22 substrates tested glucose, fructose, mannose, galactose, and lactose stimulated this endogenous respiration by 50% or more. Maltose gave a stimulation between 10% and 25%. Formate, acetate, lactate, citrate, and succinate were reported to stimulate respiratory activity less than 10%. Pyruvate was without effect.

Under anaerobic conditions CO_2 was released from a bicarbonate medium with a $q^{N_2}_{CO_2}$ of 325. This was increased by addition of exogenous substrate.

Of inhibitors studied, the following gave the indicated per cent inhibition of endogenous aerobic and anaerobic activity.

Inhibitor	Conc.	% inhibition aerobic	anaerobic
Sodium fluoride	0.04 M	8	14
Iodoacetate	0.01 M	80	--
"	0.001 M	61	--
"	0.0003 M	--	88
Hydroxylamine	0.01 M	10	--
2,4-DNP	0.001 M	12	--
8-OH-quinoline	0.001 M	--	10
Sodium azide	0.02 M	--	26
Arsenite	0.02 M	--	22
KCN	0.01 M	--	25

Arsenite, malonate, 8-hydroxy-quinoline, and azide stimulated aerobic metabolism rather than inhibiting it.

Studies on glycogen utilization and formation showed that under aerobic conditions the organism increased its glycogen reserves when incubated with glucose, galactose, or lactose. With maltose a net decrease of glycogen resulted. Anaerobically the same was observed, but in the presence of lactose and galactose the organism stored very little intracellular polysaccharide. This material was presented by Ryley (loc. cit.) to show that data on oxygen uptake or CO_2 production in the presence of exogenous carbohydrate cannot be considered as representative of "utilization" of these sugars, if by that term is meant complete immediate breakdown to CO_2 and H_2O (or to acid end products) to gain cell energy. Andrews and von Brand (1938), in studies on T. foetus, had found that the rate of glucose consumption was not uniform throughout growth, being high during periods of active multiplication and low during periods of decreasing population.

Analysis of the medium after anaerobic glycolysis by T. foetus (Ryley, 1955a) revealed succinic acid and acetic acid as the major products. CO_2 was assimilated (fixed) in the process, and considerable hydrogen was also produced.

With cell-free preparations (made in a Mickle disintegrator) evidence was obtained for the presence of

amylase, maltase, phosphorylase, hexokinase, phosphoglucomutase, ketoisomerase, a metal-activated aldolase, and a system oxidizing triosephosphate which was DPN dependent.

Wirtschafter (1954) presented evidence for the existence of hexokinase and aldolase in T. vaginalis, and indicated that two strains of the organism differed in their content of these enzymes.

Baernstein (1955) characterized the aldolase of T. vaginalis in sonic homogenates of the organism and found it to be activated by cobaltous and ferrous ions, but inhibited by the trivalent forms of these metals.

A short abstract appeared (Read, 1955) indicating that work had been done on the comparison of the metabolic activities of T. vaginalis and T. gallinae and, although quantitative differences were implied, no data were given in the abstract. It was stated that T. gallinae was found to oxidize all Krebs cycle intermediates, the first report of this activity for these flagellates, and in contrast to the statement by Willems, et al. (1942) that succinic acid is not utilized by this organism.

Recently, Wirtschafter and Jahn (1956) and Wirtschafter, Saltman, and Jahn (1956) reported on the aerobic and anaerobic pathways of carbohydrate degradation in T. vaginalis. Phosphoglucomutase, alpha-glycerophosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogen-

ase were demonstrated in homogenates of this organism. Tricarboxylic acid cycle intermediates other than malate were not utilized. Glucose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, 2-phosphoglyceric acid, 3-phosphoglyceric acid, and phospho-enol pyruvate were identified by chromatography of reaction mixtures. A radioactive product which incorporates a C^{14} label from pyruvate was found but not identified.

All the foregoing authors have failed to give specific details about the age of the culture used, and therefore, comparison of work was difficult unless it was assumed that metabolic activity was relatively constant over a period of about 24-96 hours. That this assumption is not valid has recently been indicated by Doran (1956a). He found that the q_{O_2} of T. foetus decreased from a high of 277 at 20 hours to 190 at 48 hours with much variation between. A further decrease to 65 was observed with organisms 120 hours old. Acid formation from glucose also decreased with time. Variations in metabolic activity in T. vaginalis were noted by Read and Rothman (1955) who attributed the differences to the quality of the media, and the age and strain of the inoculum.

Using 48-hour cultures Doran (1956b) studied aerobic carbohydrate metabolism of T. foetus and the three forms of T. suis (small cecal form, large cecal form, and nasal form). His results regarding substrate oxidation were as

follows:

Substrate	*T. foetus*	small cecal	large cecal	nasal
Glucose	*x	-	x	x
Galactose	*x	o	x	x
Mannose	*x	-	x	x
Fructose	*x	o	x	x
Maltose	x	x	x	x
Lactose	o	o	x	x
Raffinose	o	o	x	x
Trehalose	x	o	x	x
TCA interme d.	o	o	o	o

x-utilized o-not utilized *-utilized at rate 3 times faster than others.

In further studies iodoacetate and arsenite were shown to inhibit oxygen uptake by all forms. *T. foetus* and the nasal form of *T. suis* were also inhibited by fluoride and 8-hydroxy-quinoline. Cyanide, azide, arsenate, 2,4-dinitrophenol, and malonate showed no antagonistic action.

Read (1953) reported that anaerobic gas production by *T. vaginalis* at pH 5.8 was inhibited 70% by 10^{-3} M potassium cyanide, but was not affected under aerobic conditions. He also noted that carbon monoxide inhibited anaerobic gas production completely in the dark, but illumination of the cells reversed the effect. The conclusion reached was that an iron porphyrin was involved in anaerobic metabolism - the first time this has been reported for animals. Suzuoki and Suzuoki (1951a) have reported evidence for the presence of cytochrome b in *T. foetus*,

while Ryley (1955a) was unable to find any cytochromes. Riedmüller (1936) also failed to detect any cytochrome pigments.

Purification and physico-chemical characterization of the intracellular glycogens of *T. foetus* and *T. gallinae* have been performed by Manners and Ryley (1955). Feinberg and Morgan (1953) have also described the isolation of a polyglucose from *T. foetus*. These workers also found another polysaccharide with an attached amino acid moiety. This substance contained rhamnose, fucose, xylose, galactose, and glucosamine plus ten amino acids. This latter substance was shown to be non-antigenic in rabbits.

It is evident from the foregoing review that much basic research is yet necessary to elucidate the complex biochemical activities of the trichomonads. Additional and confirmative data on standard conditions, substrate utilization, inhibitors, and enzyme content is urgently needed, especially from species other than *T. foetus* and *T. vaginalis*.

THE PROBLEM

The present research was undertaken to investigate some aspects of carbohydrate metabolism in four trichomonads. After much preliminary work, the problem resolved itself into the following component parts:

(a) study of growth of the organisms in bacteria-free culture under usual laboratory conditions;

(b) determination of changes in the metabolic activity of the organisms during growth in artificial media, and standardization of conditions for manometric study;

(c) investigation of substrate utilization and effects of various inhibitors;

(d) investigation of the production of hydrogen by these organisms;

(e) analysis of cell-free sonic homogenates for some enzymes likely involved in intermediary metabolism;

(f) comparison of the organisms on the basis of any differences which might be found to exist, in such items as respiratory activity, enzyme content, effect of inhibitors, and accumulation of end products.

MATERIALS AND METHODS

Organisms - The four organisms used in this study were obtained in bacteria-free culture from earlier work (Sanborn, 1955) in this department. They had been isolated originally from their appropriate animal hosts in the veterinary clinic of Michigan State University, and had been carried in culture in a modified CPLM* medium since.

As listed earlier, the organisms used were Tritrichomonas foetus, Pentatrichomonas gallinarum, and nasal and fecal forms of Trichomonas suis.

For the various experiments appropriate dilutions of the cultures were made and the organisms counted in a hemacytometer chamber as for white blood cells. The usual dilution was 1:10. Then, if the four corner squares in the chamber are used, multiplication of the count obtained by 25,000 gives the number of organisms per ml.

Cultural methods - Several media were tried in an effort to find one suitable for the present work. The complexity of the CPLM medium rendered it difficult to make in large quantities, and its agar content made it useless for harvesting large quantities of organisms by centrifugation.

Several commercially-available dehydrated culture media were found to support good growth of these protozoa when supplemented with serum. The serum used was dehydrated Beef Blood Serum (Difco) which was made in a 20%

*-cysteine-peptone-liver-maltose

(of normal) concentration, filtered through gauze and paper, sterilized by gravity passage through a Seitz filter, and added to all cultures so as to give a final concentration of 1-2%. Adequate growth was obtained at this concentration and the organisms did not show the high rate of endogenous respiration reported by others.

Three types of cultures were used routinely in this study. Stock cultures were maintained in 20 ml amounts of Difco Brewer Thioglycollate Medium in 25 x 150 mm test tubes. Transfers were made weekly a nd the cultures incubated at 37° C for about 36 hours. They were then placed at room temperature where the cultures remained viable for about 3 weeks. Special preparatory cultures were planted prior to inoculation of larger amounts of media for bulk growth of the organisms. These were made by inoculating 20 ml of NIH Thioglycollate Broth (Case or Difco) in 25 x 150 mm test tubes with about 1.5 ml of stock culture and incubating about 36 hours at 37° C. The contents of one tube was then inoculated into a 250 ml Erlenmeyer flask containing 100 ml of the same (NIH) medium. This culture was incubated the required length of time (12-72 hours) and harvested by centrifugation in 50 ml amounts at 1500 RPM (approx. 500 RCF) in an International No. 2 horizontal centrifuge. The sedimented organisms were washed twice with 0.9% NaCl, and finally taken up with an appropriate amount of 0.9% NaCl or (for sonic

extract preparations) 0.9% KCl. In most cases where whole cells were used the dilution was such that each ml contained approximately 50 million (5×10^7) flagellates.

Manometry - The experiments on respiration and fermentation were conducted in accord with standard manometric practice (Umbreit, 1949) at 37° C in a Precision Warburg 20-unit respirometer with a shaking rate of 120 per min. Gas atmospheres were altered as desired by gassing the flasks while in the bath through a 10-place gassing manifold. Gases used were nitrogen, 5% CO_2 - 95% N_2, and hydrogen.

Cell-free extracts - These were prepared by harvesting cells as described above (after 36 hours of incubation), diluting to approximately 10^8 cells per ml with ice-cold 0.9% KCl, and subjecting the suspension to vibrations in the chilled (2° C) cup of a Raytheon 10 kc Sonic Oscillator for 7 minutes. This treatment time was sufficient to rupture practically all the cells, the cloudy, almost opaque suspension being changed to a faintly opalescent solution. The cellular debris was removed by centrifuging lightly and the extract was immediately frozen in 5 ml amounts at -20° C.

Chemical methods - Succinic acid was assayed manometrically as described by Umbreit (1949) using a succinoxidase preparation from fresh pig heart. Pyruvate was measured by the procedure of Lu (1939) as described in modified form

by Umbreit (1949), and lactic acid by the method of Barker and Summerson (1941). Reducing sugar was estimated by the ferricyanide method of Folin and Malmros (1929), and fructose by the procedure given by Roe (1934). Inorganic phosphate was determined by the procedure of Fiske and SubbaRow (1925). Estimation of nitrogen was by direct nesslerization after digestion in 5 N sulfuric acid and 30% H_2O_2 and neutralization with 5.5 N KOH. The general procedure as given by Umbreit (1949), including the modified Nessler's solution, was used. Digestion was on a micro-Kjeldahl digestion rack for about half the determinations; the others were digested in the oven as described.

Colorimetric determinations were made with the aid of a Bausch and Lomb Spectronic-20 spectrophotometer.

Enzyme determinations - Catalase activity was assayed manometrically by measurement of oxygen released from 10 micromoles of H_2O_2. Hydrogenase was assayed by measuring hydrogen uptake manometrically in an atmosphere of hydrogen and in the presence of 25 micromoles of methylene blue with KOH in the center well of the flask. Formic hydrogenlyase was determined by following CO_2 and H_2 formation from 25 micromoles of pyruvate under an atmosphere of nitrogen. Formic dehydrogenase activity was measured by estimating CO_2 production from 50 micromoles of formate in the presence of 25 micromoles of methylene blue under a nitrogen atmosphere.

Hexokinase activity was assayed by the manometric method of Colowick and Kalckar (1941), phosphoglucomutase by the method given by Najjar (1955), phosphohexoisomerase by measuring fructose-6-phosphate formation from glucose-6-phosphate as described by Slein (1955), and aldolase and phosphofructokinase by the "hydrazone chromogen" procedure of Sibley and Lehninger (1949). Phosphoglyceromutase and enolase activities were indicated by measuring pyruvate formation from phosphoglyceric acid in reaction mixtures as described by Vandemark and Wood (1956). The "phosphoroclastic" reaction was studied with the method of Koepsell (1955).

Dehydrogenase activities (other than formic dehydrogenase) were identified by following the change in absorbance at 340 millimicrons in the presence of specific substrate and diphosphopyridine nucleotide or triphosphopyridine nucleotide in a Beckman DU spectrophotometer, and in two cases, by the Thunberg methylene blue decolorization method.

Details regarding these procedures, and modifications thereof, are given in appropriate context.

All general chemicals were of highest purity. Metabolic intermediates were purchased from Nutritional Biochemical Corporation and from General Biochemicals, Inc. Certain special organic reagents were products of Eastman Organic Chemical Division of Eastman Kodak Company.

EXPERIMENTAL RESULTS

Growth studies - The nature of the research made it desirable that as simple a medium as possible be employed, so that it could be made in large quantities with a minimum of work. The CPLM medium of Johnson and Trussell (1943), and the STS broth of Kupferberg *et al.* (1948) were both too complex for routine use, and both contained a small amount of agar which was undesirable because of the difficulty in harvesting the cells in its presence. Therefore a search was made for commercially-available products containing tryptone or phytone, yeast extract, carbohydrate, and a reducing agent such as thioglycollate or cysteine, but no agar.

Several commercial dehydrated products* with the above requirements were found to support growth of the flagellates, but not all to the same extent. Population counts were consistently about 1.5-2.0 times higher in NIH Thioglycollate Broth (Case or Difco) than in any of the others. Counts between 3-4 million organisms per ml in 36-48 hours were obtained routinely when a sufficiently large inoculum was employed. This medium was therefore adopted and used as described earlier.

In an effort to obtain general information regarding

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* - Eugonbroth (BBL)
Brucella Broth (Albimi)
Trypticase Soy Broth (BBL)
Tryptone Soy Broth (Case)
Cystine Trypticase Agar (BBL) (tried for maintenance cultures by stab inoc.)

carbohydrate breakdown during growth in this medium, replicate cultures were inoculated with each of the various organisms and sampled periodically for 3 days. At each time, determinations were made of population, pH, titratable acidity (using 0.01 M NaOH to titrate 5 ml of culture with brom-thymol blue as the indicator), and reducing sugar. In certain samples lactic, pyruvic, and succinic acids were assayed.

Figures 1-4 show the changes occurring in the cultures of the four organisms over the three day period. The figures are not directly comparable since the original inoculum differed slightly in each case, varying from 110 to 160 thousand cells per ml of culture.

The pH was between 6.0 and 6.3 at the time of the population peak for each species. Figure 5, which represents average values from several cultures of these organisms, indicates that when the pH of the culture reached 5.0-5.2 only very few living organisms remained. These few disappeared in a few hours at this pH. (It is perhaps well to mention at this point that if the cultures are removed from the incubator at about the time of the population maximum, the organisms remain viable for longer periods of time due to their decreased reproduction rate at room temperature. Without agar, however, the population declines much more rapidly than in its presence - as in the stock cultures.)

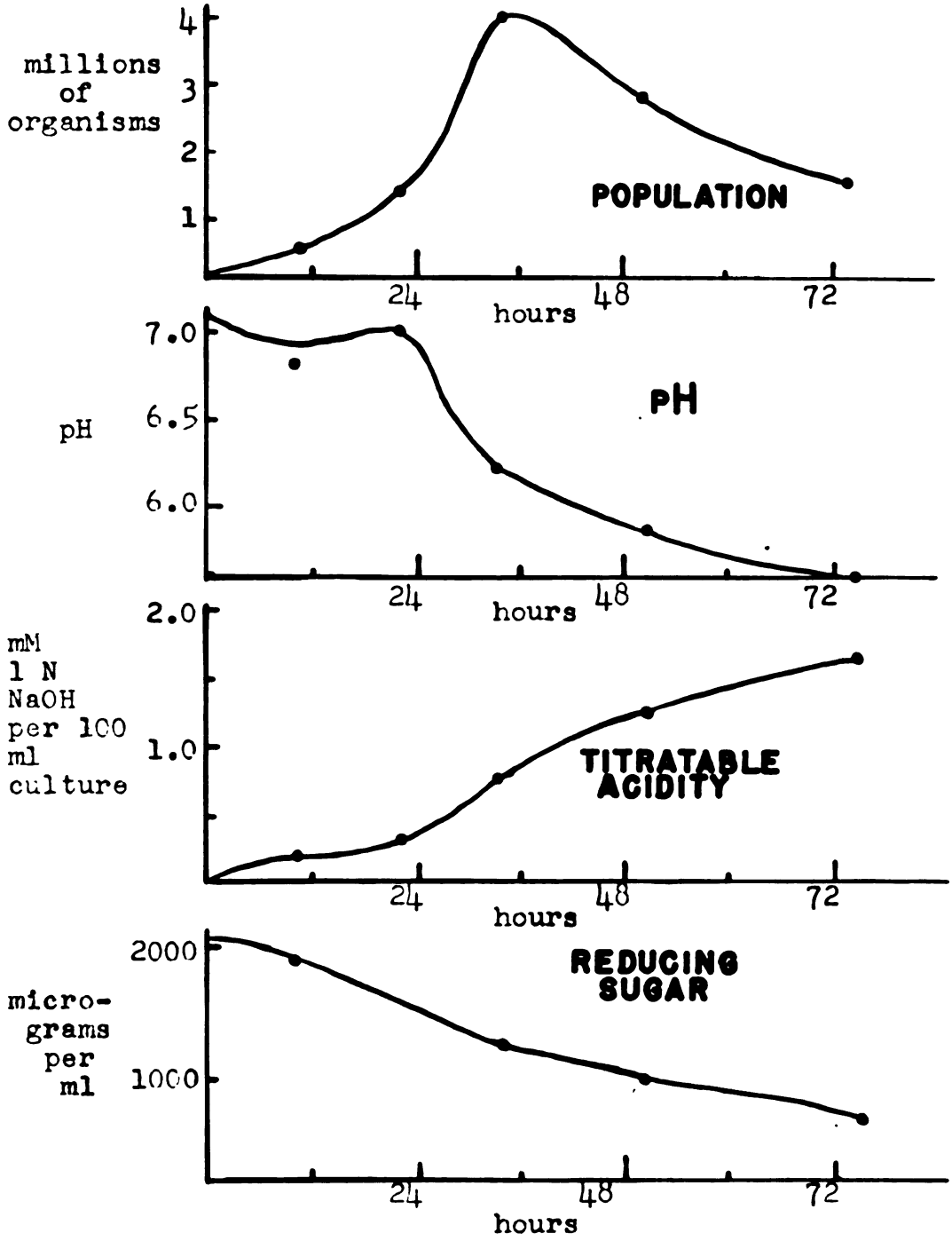


Figure 1. Changes in a Bacteria-free Culture of Tri-trichomonas foetus During Three Days Growth in NIH Thioglycollate Broth at 37 C.

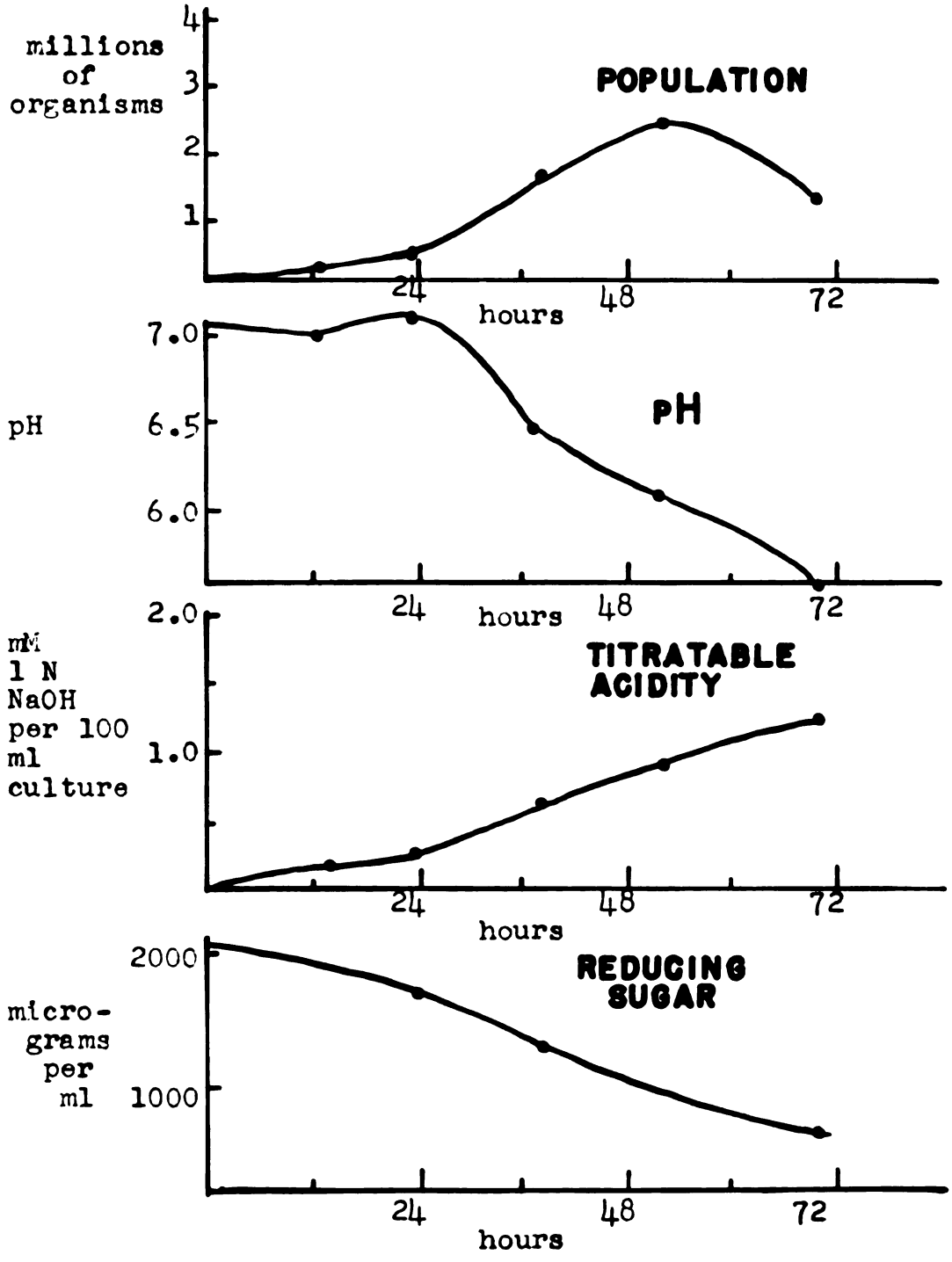


Figure 2. Changes in a Bacteria-free Culture of Pentatrichomonas gallinarum During Three Days Growth in NIH Thioglycollate Broth at 37 C.

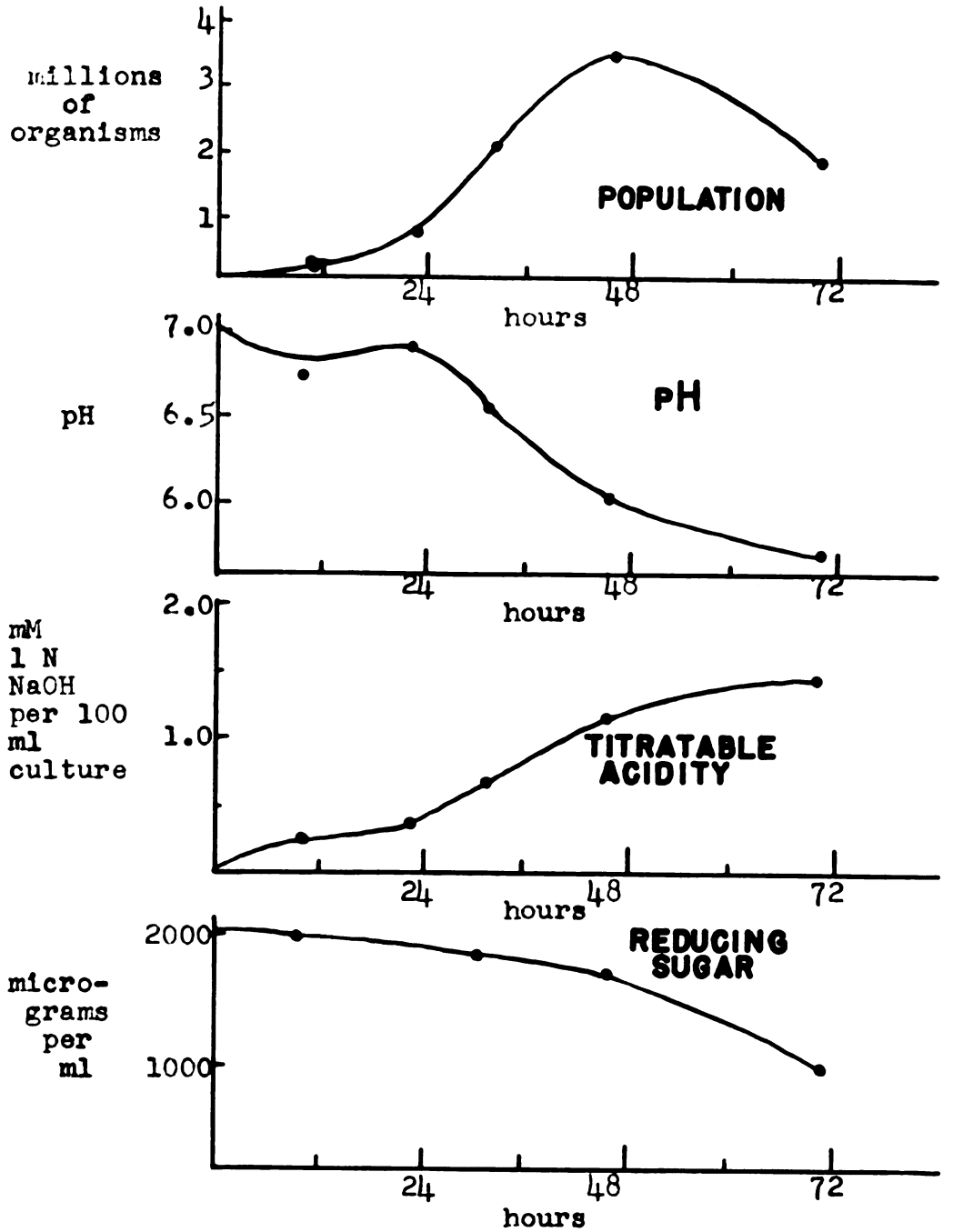


Figure 3. Changes in a Bacteria-free Culture of Trichomonas suis (fecal strain) During Three Days Growth in NIH Thioglycollate Broth at 37 C.

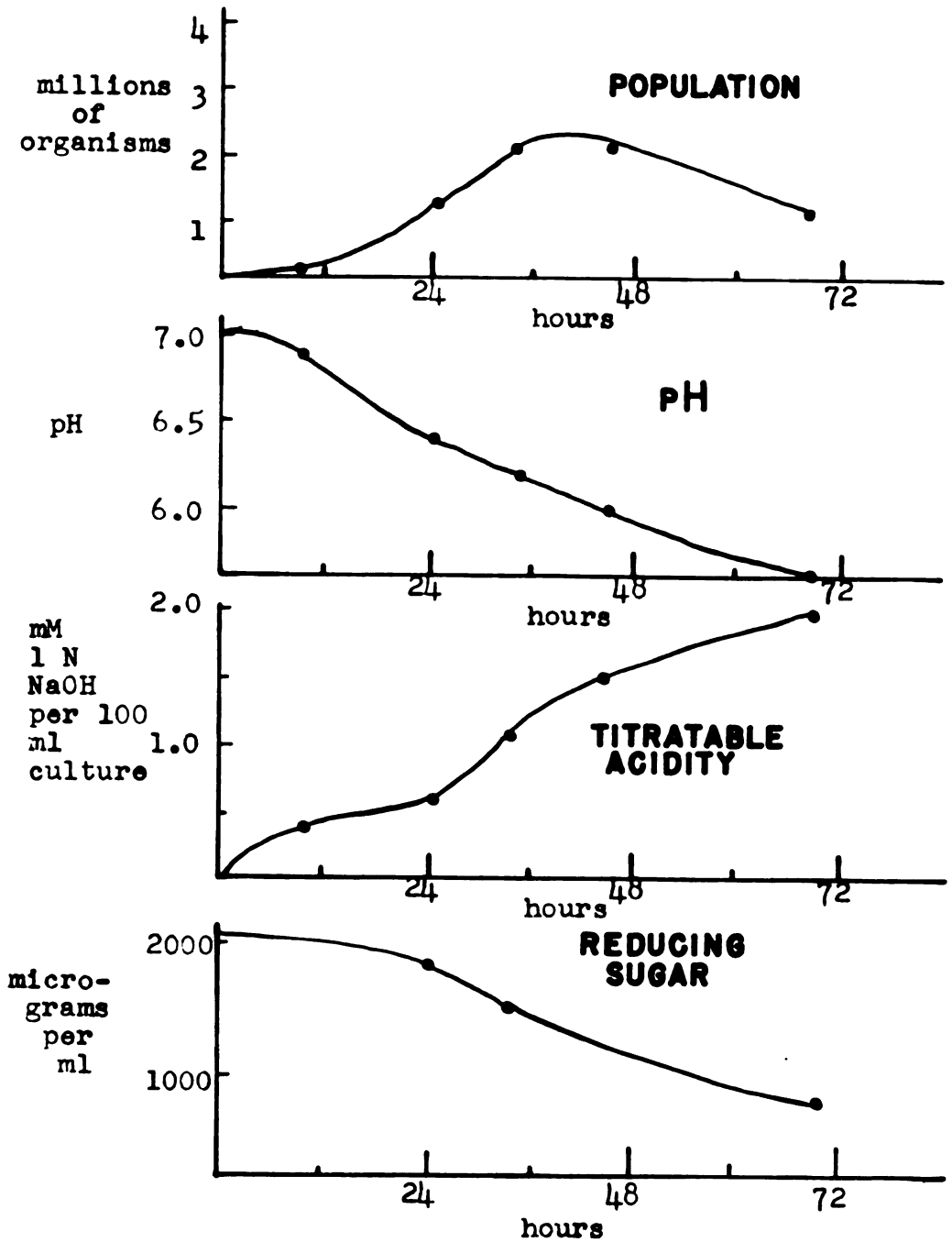
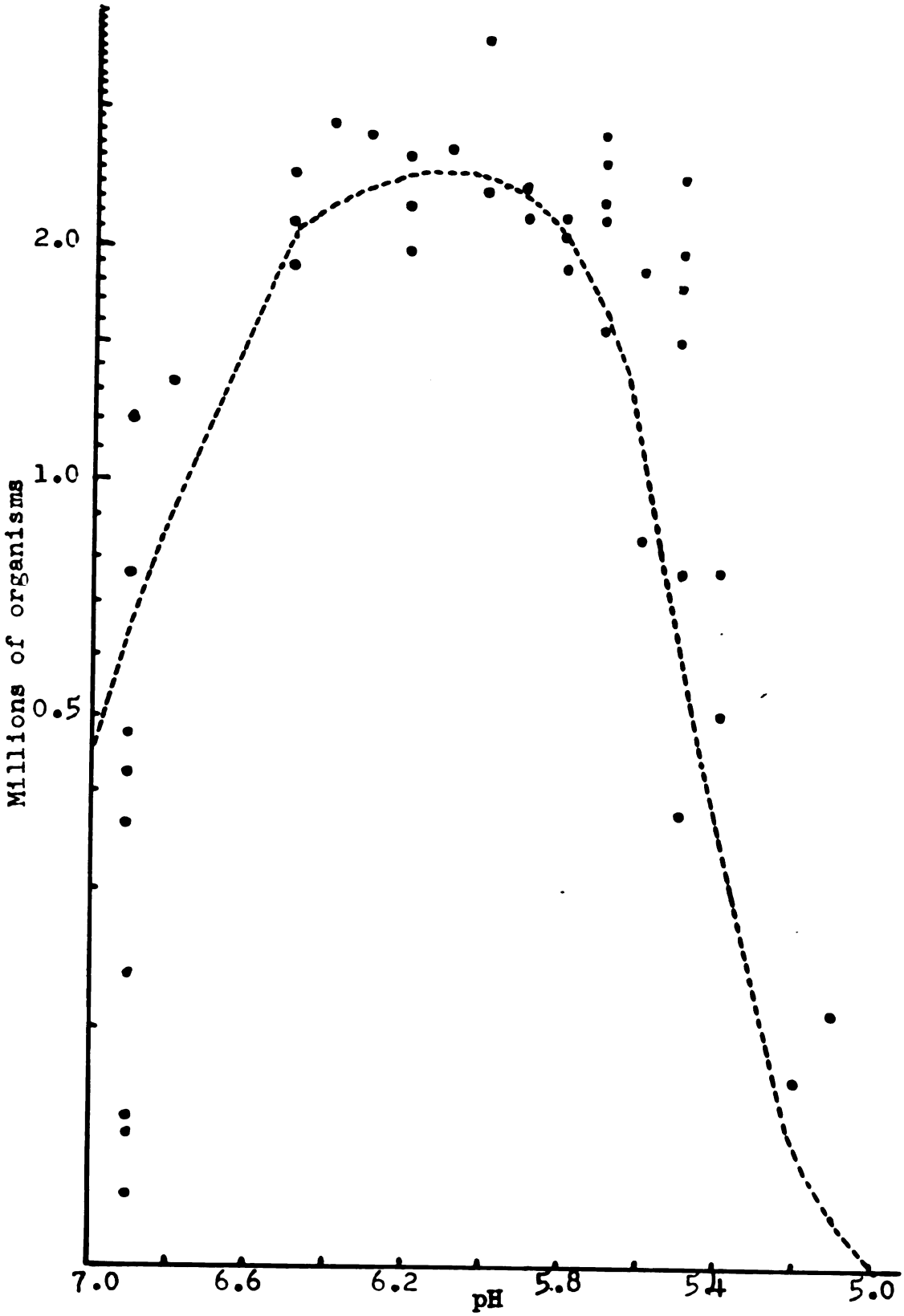


Figure 4. Changes in a Bacteria-free Culture of Trichomonas suis (nasal strain) During Three Days Growth in NIH Thioglycollate Broth at 37 C.

Figure 5. Average Relationship of pH to Population in Cultures of Four Trichomonads in NIH Thioglycollate Broth at 37 C.



That this pH drop is paralleled by an increase in titratable acidity and a decrease in the reducing sugar is apparent from Figs. 1-4. Analysis of the culture medium at various times during incubation showed that except for the fecal strain of T. suis, and to a lesser extent for T. foetus, the acidity could largely be accounted for by an increase in lactic, succinic, and pyruvic acids. This is shown (for 70-75 hrs.) in Table 1. As can be seen, succinic acid was the major acid produced by all but the fecal strain of T. suis. Of the acids shown, lactic was produced in greatest quantity by this latter organism. However, over 50% of the total acidity is unaccounted for in these analyses for T. suis (fecal). The lactic/pyruvic ratio was about 2.5 for the two swine forms, while P. gallinarum and T. foetus produced approximately equal amounts of each acid.

From these data it appears that the nasal form of T. suis produces much more acid in these cultures than the others. In an effort roughly to quantitate these differences Table 2 was prepared from the figures for total acidity and organism count at the time of maximum population. Again, from this table, it is obvious that the metabolic activities of the nasal organism under these cultural conditions result in the production of a greater amount of acidic end products than the others.

TABLE 1

Acid Production by Four Trichomonads in Bacteria-free Culture in NIH Thioglycollate Medium

Organism		Succinic Acid	Lactic Acid	Pyruvic Acid	Sum *	Titratable Acidity **
T. foetus (75 hr.)	(a)	.455	.170	.188	1.268	1.602
	(b)	56.8	10.6	11.7	79.1	---
P. gallinarum (70 hr.)	(a)	.393	.181	.188	1.155	1.158
	(b)	68.0	15.7	16.2	99.9	---
T. suis (fecal) (70 hr.)	(a)	.104	.333	.120	.661	1.394
	(b)	15.0	23.9	8.6	47.5	---
T. suis (nasal) (70 hr.)	(a)	.545	.755	.303	2.148	1.900
	(b)	57.4	39.7	15.9	113.0	---

(a) mM per 100 ml culture
(b) % of total titratable acidity
* - mM acidity titratable as monobasic acid per 100 ml culture
** - mM N NaOH per 100 ml culture

TABLE 2

Correlation of Population Density and Acid Production in Trichomonad Cultures in NIH Thioglycollate Medium

Organism	Maximum Population (millions per ml)	Titratable Acidity at this time (mM N NaOH per 100 ml)	mM Monobasic Acid per 10^8 Organisms
T. foetus	3.95	.750	189.9
P. gallinarum	2.47	.840	340.0
T. suis (fecal)	3.55	1.118	314.9
T. suis (nasal)	2.62	1.496	571.0

Changes in metabolic activity during culture - The metabolic activity of the trichomonads was seen to vary considerably depending on such factors as the buffer solution employed, the pH of the reaction mixture, and the age of the culture.

Figure 6 shows the oxygen uptake by T. foetus in the presence of various buffers with glucose as substrate. Similar results were obtained for the other organisms. The use of phosphate buffers gave in general a diminished uptake of oxygen. In the presence of tris* buffers the oxygen uptake was greater but double pH maxima were seen - one between 7.0-7.5 and the other between 8.0-9.0. This effect of tris buffers has been previously reported by Dounce, et al. (1950) in their evaluation of tests for aldolase activity.

Tris buffer of pH 8.2 was used in some of the early work but complications arising from carbon dioxide retention at this pH made it unsuitable for anaerobic studies.

Since the maximum population occurred in cultures at about pH 6.0-6.2, it was felt that measurements made near this range would have more physiological significance. Accordingly, a tris-maleate buffer** (0.05 M) of pH 6.4 was tested. This gave excellent results aerobically, and retained negligible CO_2. Therefore it was used routinely in the remaining investigations. Magnesium ion in final

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* - Tris(hydroxymethyl)aminomethane
** - Tris / maleic anhydride (TM buffer)

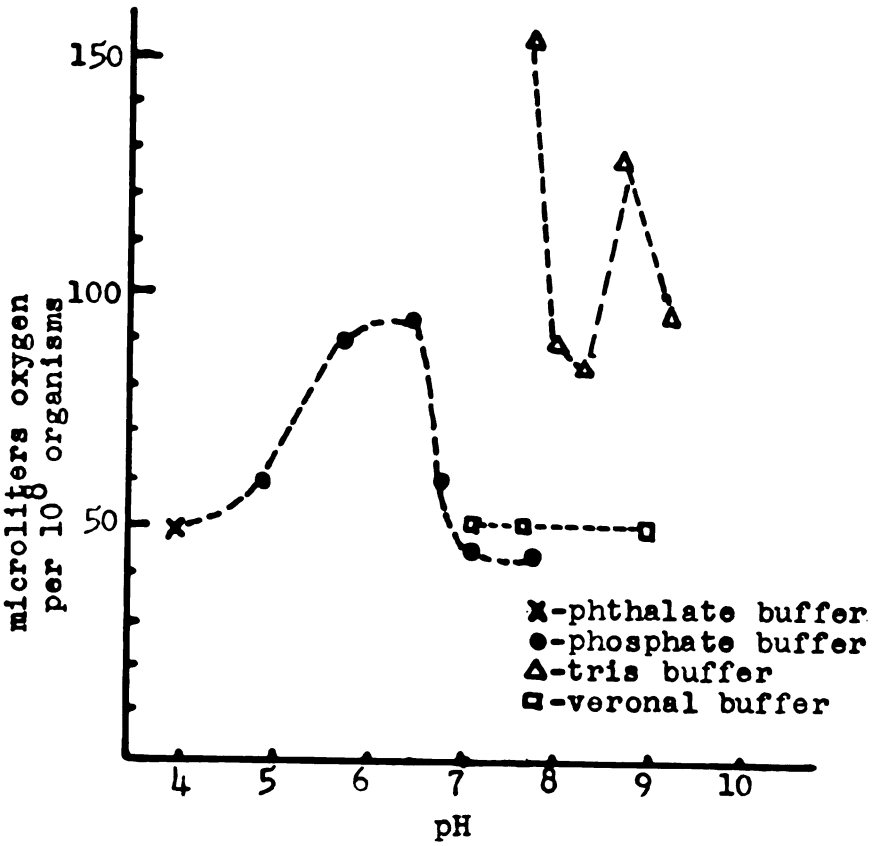


Figure 6. Oxygen Uptake in the Presence of Various Buffers by Tritrichomonas foetus Suspension.

concentration of .0025 M was added to all buffers.

Comparisons made between the TM buffer and a sodium phosphate buffer (0.05 M) at pH 6.4 showed that the oxygen uptake was less in the phosphate buffer than in TM. This resulted in higher repiratory quotients (R.Q.)* in phosphate-buffered solutions. One explanation for this could be that, for some unexplained reason, more hydrogen was produced in the phosphate medium, although there is no evidence for this. The results of the above experiments are shown in Table 3.

TABLE 3

Endogenous Respiratory Quotients of Four Trichomonads in Sodium Phosphate or Tris-Maleate Buffers at pH 6.4

Organism	Respiratory Quotients Na Phosphate	Tris-Maleate
T. foetus	1.17	0.90
P. gallinarum	1.10	0.82
T. suis (fecal)	1.30	0.99
T. suis (nasal)	1.24	0.80

*-ratio of CO_2 produced to O_2 consumed. This terminology is not entirely correct in the case of these organisms, since hydrogen is also produced (at least, under anaerobic conditions) as will be shown later. There is, however, no adequate manometric method for determining hydrogen production under aerobic conditions. The R.Q. values obtained in the presence of exogenous carbohydrate indicate that little error is introduced in this way. In culture, unless agar is included or the culture is exceptionally deep, bubbles of H_2 are rarely seen, indicating that little gas is produced aerobically.

Endogenous respiration in the TM buffer over a 72-hour culture period is shown in Figure 7. Wide variations in respiratory activity were observed between 24 and 48 hours - the period of logarithmic growth in culture. In general, all the organisms metabolized at higher rates when harvested from actively growing cultures than when taken from those in the stationary or declining phases of the growth curve.

With 50 micromoles of glucose as substrate the R.Q. of the organisms was not observed to vary appreciably over a cultural period of 12-50 hours. However, the specific respiratory activity (qO_2 and qCO_2*) was considerably different at the various times but, except for T. suis (nasal) and P. gallinarum, was highest in the 12-hour organisms. These data are presented in Table 4.

Under anaerobic conditions (5% CO_2-95% N_2) in a bicarbonate buffer at pH 6.75, it is possible to measure acid production as well as metabolic CO_2 (Umbreit, 1949).

Table 5 shows the results obtained when these two variables were measured with organisms from 12 to 48 hours old. The data are expressed as the percentage of total CO_2 evolution due to acid production and as the q_{acid} (microliters CO_2 from bicarbonate per mg cell N).

It can be seen that acid production was rather high in the experiments involving young organisms (except for

*-qO_2--microliters O_2 per mg cell N
qCO_2-microliters CO_2 per mg cell N

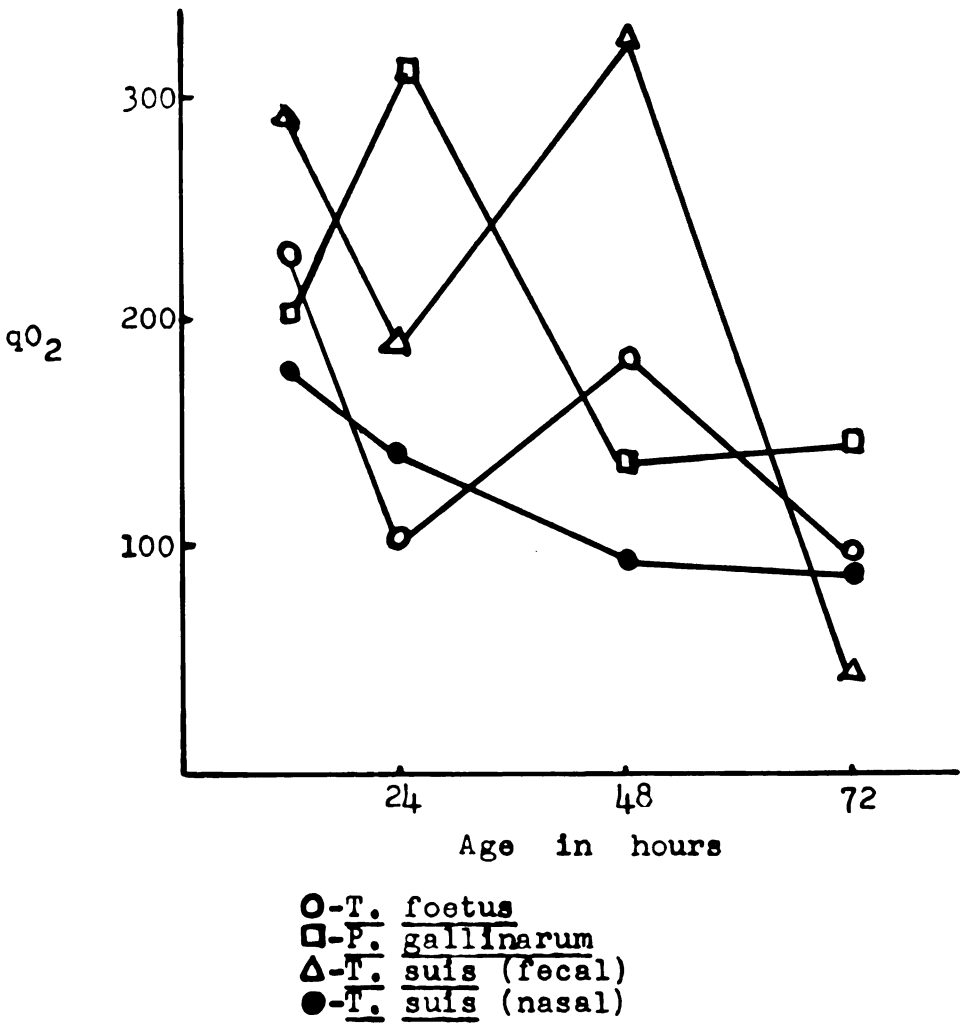


Figure 7. Endogenous Respiration of Trichomonads Harvested at Various Times During Growth in NIH Thioglycollate Broth at 37 C.

TABLE 4

Respiratory Activity of Four Trichomonads During 50 Hours of Growth in Bacteria-free Culture as Measured Manometrically in the Presence of 50 Micromoles Glucose

Organism		AGE IN HOURS					
		12	20	30	40	46	50
T. foetus	(a)	691.4	245.5	250.2	405.5	247.9	
	(b)	684.1	165.4	216.4	354.6	191.7	
	(c)	0.99	0.67	0.87	0.87	0.77	
P. gallinarum	(a)	203.6	176.8	223.3	287.9		240.3
	(b)	156.4	167.9	250.2	280.6		269.4
	(c)	0.84	0.95	1.12	0.97		1.12
T. suis (fecal)	(a)	639.5	304.4	226.8	255.8	394.5	
	(b)	639.0	252.2	195.6	206.4	335.5	
	(c)	1.00	0.83	0.86	0.81	0.85	
T. suis (nasal)	(a)	347.4	214.8	385.6	660.6		365.1
	(b)	330.2	232.2	307.8	580.3		391.9
	(c)	0.87	1.08	0.80	0.88		1.07

(a) qO_2
(b) qCO_2
(c) R.Q.

TABLE 5

Acid Production by Four Trichomonads in Bicarbonate Buffer at pH 6.75 in the Presence of 50 Micromoles Glucose

Organism	AGE IN HOURS 12	20	30	40	48
T. foetus	(a)82.5	57.9	53.8	72.4	100
	(b) 734	483	435	301	582
P. gallinarum	(a)73.1	46.0	65.9		100
	(b) 693	344	370		352
T. suis (fecal)	(a)42.3	43.3	67.9	95.1	100
	(b) 577	423	474	250	860
T. suis (nasal)	(a)80.7		87.7	88.3	100
	(b) 801		648	513	281

(a) % CO_2 attributed to acid production

(b) q_{acid}

T. suis (fecal) at 12 hours.) It dropped during the actively growing phase and then increased again until by 48 hours practically 100% of the CO_2 produced was due to acid formation. The q_{acid}, on the other hand, declined with time in these experiments.

Substrate utilization and inhibitors - Twenty-one compounds were tested for their ability to stimulate the respiration of the organisms in this study. They can be divided into three groups: (a) intermediates of the Meyerhof-Embden glycolytic pathway, (b) tricarboxylic acid cycle intermediates, and (c) mono- and di-saccharide sugars. Table 6 shows the results obtained when the substrate (usually 20 micromoles) was added to the organisms from the sidearm of a standard Warburg vessel. The figures given are ratios of the oxygen uptake in the presence of substrate to the endogenous oxygen uptake. It can be seen that most of the compounds tested had no stimulatory effect; in fact, many of them were decidedly inhibitory. Only the sugars and, in a few cases, formate, pyruvate, lactate, and malate stimulated oxygen uptake.

Of the sugars, those which stimulated respiration the most were generally glucose, mannose, and fructose. Galactose was included in this group for all except T. gallinarum, which also did not oxidize fructose very rapidly. The disaccharides were less effective in

TABLE 6

Effect of Various Substrates on the Respiration of Four Trichomonads

Substrate	T. foetus	P. gallinarum	T. suis (F)	T. suis (N)
Glucose	1.50	1.60	2.01	2.66
Mannose	1.43	1.36	1.83	2.22
Fructose	1.62	1.30	1.72	2.45
Galactose	1.47	1.18	1.60	2.59
Maltose	1.14	1.12	0.99	1.58
Lactose	1.05	0.89	1.19	1.63
Sucrose	1.23	0.96	1.08	1.03
Glucose-1-phosphate	--	--	0.75	0.84
Hexose diphosphate	--	1.07	1.00	1.29
Phosphoglycerate	--	0.91	0.61	0.82
Pyruvate	0.91	1.35	0.60	0.58
Lactate	0.99	0.77	1.30	0.97
Formate	0.93	1.45	0.80	0.78
Oxalacetate	0.82	0.63	0.90	0.76
α-ketoglutarate	0.70	0.86	0.59	0.80
Malate	0.78	1.40	0.82	0.83
Fumarate	0.83	0.84	0.87	0.68
Succinate	0.88	0.76	0.77	0.75
Citrate	0.65	0.95	0.71	0.44
Acetate	1.04	0.78	0.80	1.08
Glycerol	0.83	0.87	0.84	0.76

Figures given are the ratio of oxygen uptake in the presence of the substrate to the oxygen uptake in its absence.

increasing the rate of oxygen uptake, undoubtedly due to the necessity for hydrolysis before they could be utilized. Lactose and sucrose failed to stimulate the respiration of P. gallinarum. All of the sugars were utilized at greater rates by the nasal T. suis.

Ryley (1955a) indicated that a net synthesis of intracellular glycogen took place during utilization of exogenous carbohydrate. In an attempt to repeat parts of this work, the glycogen content of the cells used to measure aerobic sugar utilization was determined before and after each experiment. The results (shown in Table 7) indicated that for most sugars used under aerobic conditions by T. gallinarum and the two forms of T. suis there was a synthesis of intracellular polysaccharide. However, it was not possible to demonstrate this with our strain of T. foetus, except for maltose. The results for that organism are in direct opposition to those of Ryley, who reported that maltose was the only carbohydrate which did not cause intracellular synthesis. The endogenous controls for these experiments for T. foetus were unsatisfactory because of accidental contamination with sugar, so therefore the results obtained for this organism are in all probability in error.

The low respiratory rates given for formate, pyruvate, and lactate for all but P. gallinarum may be due to the production of hydrogen which would obscure the respiratory

TABLE 7

Intracellular Glycogen Change in Four Trichomonads when Incubated 1 hour with 50 Micromoles of Various Sugars

Substrate	NET GLYCOGEN CHANGE (as micromoles glucose equivalent per milligram cell nitrogen)			
	T. foetus	P. gallinarum	T. suis (F)	T. suis (N)
Glucose	-0.3	±2.5	±3.2	±0.8
Mannose	-0.8	±1.9	±0.4	±2.4
Fructose	-1.0	-0.5	-2.9	±3.7
Galactose	-1.2	±1.4	±2.4	-0.7
Maltose	±0.4	±1.3	±3.2	±1.0
Lactose	-1.1	±0.2	±3.1	±1.0
Sucrose	-0.2	±2.1	±3.0	±1.1

picture. This is discussed in a later section.

Ten inhibitors of various enzymatic reactions were tested for their effect on aerobic metabolism of the four organisms. The results are given in Table 8.

The most potent antagonist was iodoacetate, a powerful inhibitor of glycolysis. Fluoride, another glycolysis inhibitor, was only slightly effective in high concentrations. Other agents, such as 8-hydroxy-quinoline and 2,4-dinitrophenol with P. gallinarum, and hydroxylamine with P. gallinarum and the fecal T. suis, gave slight inhibitions. Arsenate was appreciably stimulatory with the fecal T. suis and T. foetus, and 2,4-dinitrophenol also stimulated these two as well as the nasal pig form. Arsenite showed very slight inhibition only with P. gallinarum and T. foetus. Malonate did not affect respiration-another indication that the Krebs tricarboxylic acid cycle is not functional in these organisms.

All four organisms were found to possess catalase activity. Incubation of cell suspensions containing 10^8 organisms with 10 micromoles H_2O_2 resulted in a rapid evolution of oxygen. The reaction was complete within 10 minutes. With 2.5×10^7 ($\frac{1}{4}$ as many) organisms the reaction was slower, requiring 30 minutes for completion with the nasal T. suis and P. gallinarum and 45 minutes with the fecal T. suis and T. foetus.

TABLE 8

Effect of Various Inhibitors on the Respiration of Four Trichomonads in the Presence of 250 Micromoles Glucose

Inhibitor	Conc.(M)	*T. foetus*	*P. gallinarum*	*T. suis*(F)	*T. suis*(N)
Arsenate	.01	1.56	0.87	1.52	1.18
	.001	1.16	1.01	1.19	1.01
	.0001	0.99		0.95	
Arsenite	.01	0.94	0.91	0.99	1.14
	.001	0.87	0.88	0.95	
Cyanide	.1	0.62			0.99
	.01	1.04	0.94	1.09	0.92
Fluoride	.1	0.78	0.90		
	.01	1.00	0.85	1.05	1.08
Hydroxylamine	.1	0.81		0.69	
	.01	1.06	0.87	0.84	1.03
Iodoacetate	.01	0.40	0.20	0.34	0.13
	.001	0.81	0.24	0.39	0.30
	.0001	0.96	0.80	0.83	
Malonate	.01	1.02	1.11	0.99	1.02
2,4-DNP	.001	1.26	0.77	1.44	1.20
	.0001	1.21	0.94	1.05	1.00
8-OH-Q	.01		0.88		
	.001	1.18		1.11	1.01
Azide	.1				0.96
	.01	1.01	1.00	0.82	
	.001			0.89	

Figures given are the ratio of oxygen uptake in the presence of inhibitor to the oxygen uptake in its absence.

Inhibitors were made up to concentration shown in 10% glucose and 0.5 ml of this solution added to cells from flask sidearm after equilibration at 37° C.

Carbon dioxide production from bicarbonate under 95% N_2 - 5% CO_2 was strongly inhibited in all organisms by iodoacetate, but not by arsenite or fluoride. This is shown in Table 9.

TABLE 9

Effect of Three Inhibitors on the Anaerobic Metabolism of Four Trichomonads in the Presence of 50 Micromoles of Glucose

Organism	INHIBITORS		
	Arsenite .007 M	Iodoacetate .01 M	Fluoride .02 M
T. foetus	1.21	0.22	1.43
P. gallinarum	1.07	0.24	1.27
T. suis (fecal)	1.09	0.33	1.01
T. suis (nasal)	1.18	0.22	1.12

Figures given are the ratio of CO_2 production in the presence of inhibitor to the CO_2 production in its absence.

Hydrogen production by these trichomonads - As previously mentioned, several authors have reported that an alkali-insoluble gas is produced by cultures of T. foetus and T. vaginalis. The gas has been rather conclusively identified as hydrogen.

The four organisms employed in this study were all observed to produce large amounts of gas when grown in culture media containing small amounts (0.05%) of agar, or in media without agar if the fluid was more than about one inch in depth. This gas production was evidenced by evolution of many gas bubbles when the agar-containing

ulture was agitated with a pipette, or by the accumulation f a froth on the surface of the broth medium. Shaking of either type of culture resulted in the release of more gas. These characteristics became less pronounced with increasing age of the culture, disappearing almost entirely by about 72 hours.

The figures given in Table 10 represent a series of experiments wherein the evolution of this gas (calculated as H_2) from a glucose substrate by suspensions of organisms of different cultural ages was determined manometrically under a nitrogen atmosphere. It can be seen that production of this gas was greatest by organisms from the 12-hour culture with a general decrease from then on. A slight, unexplained, increase occurred with 36-hour organisms.

TABLE 10

Evolution of an Alkali-insoluble Gas (Hydrogen) From a Glucose Substrate by Trichomonad Suspensions Under Anaerobic Conditions

Organism	Age of Culture in Hours			
	12	24	36	48
T. foetus	100	37.4	47.3	17.3
P. gallinarum	100	30.9	60.3	50.5
T. suis (fecal)	100	27.1	20.8	40.9
T. suis (nasal)	100	36.2	69.8	59.2

Figures for gas evolution are expressed as per cent of the twelve-hour level.

It has been postulated (Umbreit, 1952) that tricho-
ad hydrogen evolution is due to the action of a formic
rogenlyase enzyme system, although this has not been
ven. Also, Ryley (1955a) thought it was "...likely
at T. foetus contains a phosphoroclastic system similar
that of Clostridium butyricum", wherein hydrogen,
arbon dioxide, and acetyl phosphate are formed from
yruvate without formate intervention.

Thirty-hour organisms showed a slight increase in
ydrogen production when supplied with 50 micromoles of
lactate, pyruvate, or formate under anaerobic conditions
(N_2), as shown in Table 11.

TABLE 11

Stimulation of Hydrogen Production of Trichomonad Suspensions by Three Substrates

Organism		Lactate	Pyruvate	Formate
T. foetus	(a)	6	0	67
	(b)	5.2	-	62.7
P. gallinarum	(a)	59	10	30
	(b)	34.4	5.9	17.4
T. suis(fecal)	(a)	0	7.4	0
	(b)	-	20.1	-
T. suis(nasal)	(a)	0	19	0
	(b)	-	19.9	-

(a) per cent stimulation of endogenous rate
(b) microliters H_2 per mg cell nitrogen (qH_2)

The actual amounts of hydrogen produced were rather small as can be seen from the table. In general, it appeared that more CO_2 was produced than H_2, but determin-

ons of this gas in combination with hydrogen under se conditions have been shown to be erroneous (Ryley, 55a) since CO_2 is fixed by the organisms. Some of the esent experiments also showed a net assimilation of CO_2, nen standard methods were used for calculating the exchange f two gases.

Formic hydrogenlyase is reported to be an adaptive enzyme in such organisms as Salmonella (Stokes, 1956, and others). It is best formed by organisms grown under reducing conditions (deep broth) in the presence of glucose and a mixture of amino acids or other nitrogen source. Cells which do not form this enzyme during growth can often be adapted in the presence of glucose, formate, and amino acids, and the adaptation followed by manometric methods.

Figure 8 shows the results of an experiment in which cells were incubated with glucose, formate, and casein hydrolysate (Casamino Acids, Bacto) under a nitrogen atmosphere. KOH was present in the center well of the flasks to absorb CO_2. T. foetus and P. gallinarum showed a typical adaptation curve after a 15-minute lag. T. suis (fecal) showed no lag period and the nasal form failed to show any activity. The curves for T. foetus and P. gallinarum decreased after 30 minutes, long before the theoretical amount of H_2 had been liberated from the formate.

Formic hydrogenlyase has often been identified as

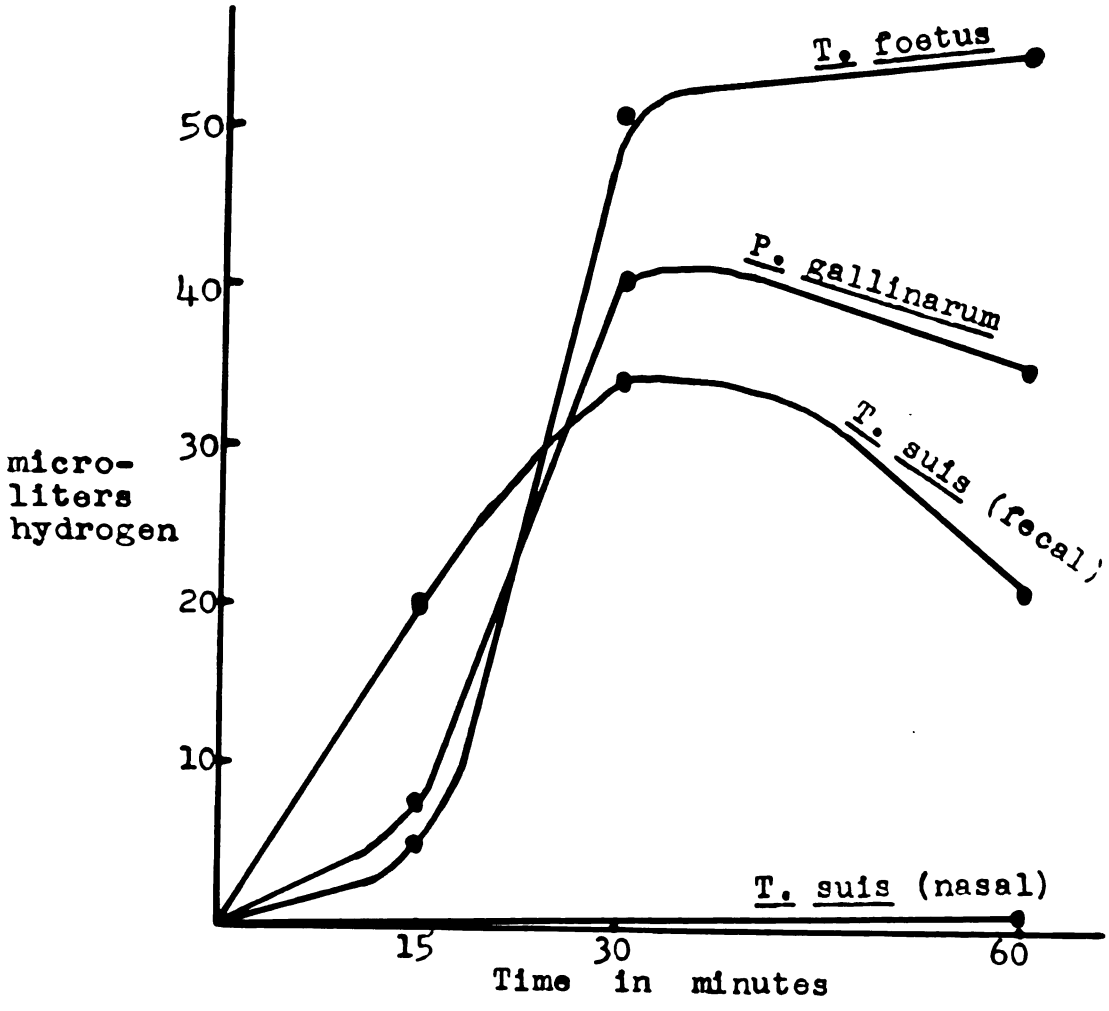


Figure 8. Response of Four Trichomonad Suspensions to Formate, Glucose, and Casein Hydrolysate Under Nitrogen Atmosphere (Hydrogenlyase Adaptation-?)

Warburg flask contained:
1 ml organisms (5×10^7)
0.5 ml TM buffer pH 6.4
0.5 ml 0.1 M Na formate
0.5 ml 3% Casamino Acids (Difco)(sidearm)
0.3 ml 10% glucose (sidearm)
0.2 ml 20% KOH (center well)

mixture of two enzymes - formic dehydrogenase, which talyzes the breakdown of formic acid to $2H^+ + 2e^-$, and drogenase, which converts these products to gaseous ydrogen. (Organisms have, however, been found which pparently either produce hydrogen without having one of these enzymes, or which have both but yet do not produce gas. Consequently the status of these enzymes is still not completely clear).

Attempts were made to demonstrate the actions of these enzymes in suspensions of the four trichomonads.

Observation of gas evolution from formate under nitrogen in the presence of methylene blue as a hydrogen acceptor gave the results shown in Figure 9, indicating a rather weak but definite formic dehydrogenase activity.

Figures 10 and 11 show the results obtained when the cells were incubated with no exogenous substrate in the presence of methylene blue or sodium fumarate in an atmosphere of hydrogen. P. gallinarum took up the most hydrogen with the nasal T. suis showing the greatest activity of the other three. For these three fumarate was an even poorer acceptor for hydrogen, while with P. gallinarum, it acted slightly better than methylene blue.

In order to investigate Ryley's suggestion that a "phosphoroclastic" split of pyruvate perhaps occurred in these organisms, the assay procedure of Koepsell (1955)

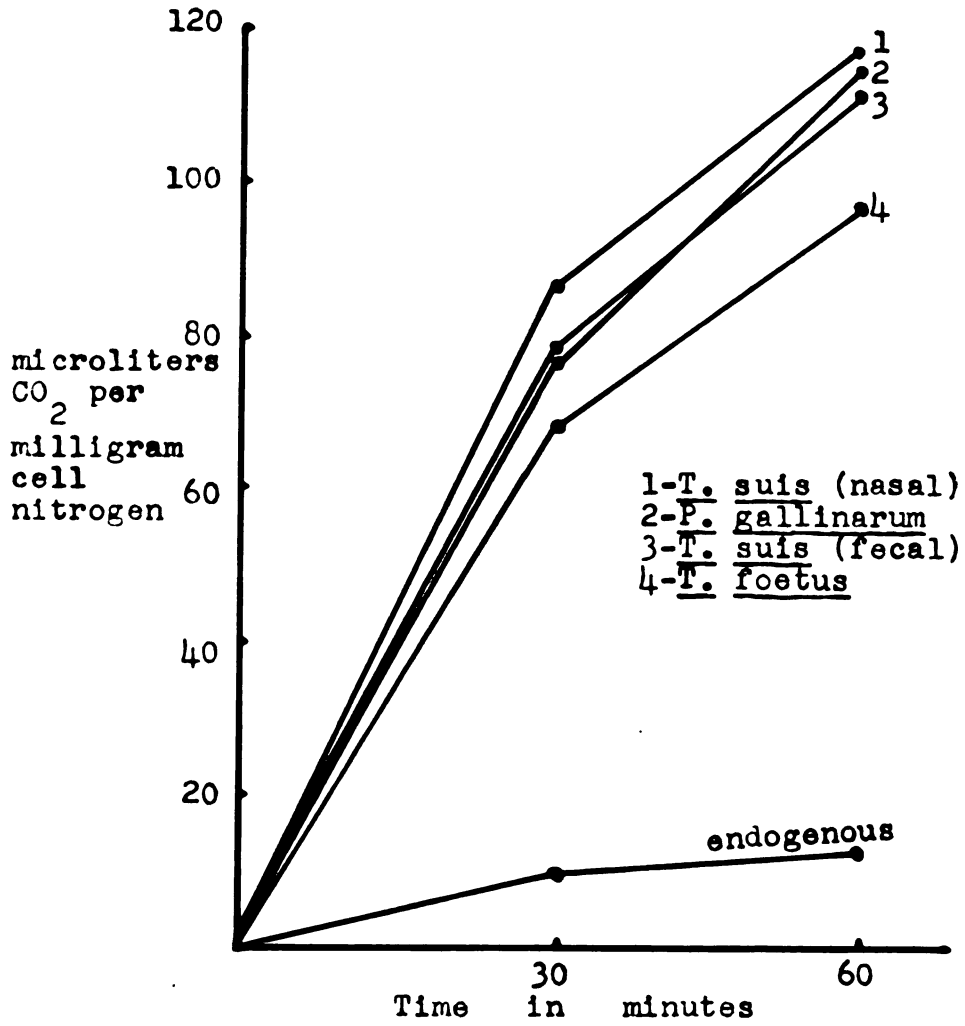


Figure 9. Formic Dehydrogenase Activity of Suspensions of Trichomonad Cells

Warburg flask contained:

1 ml organisms (5×10^7)
1 ml TM buffer pH 6.4
0.5 ml 0.05 M methylene blue
0.5 ml 0.1 M sodium formate (sidearm)

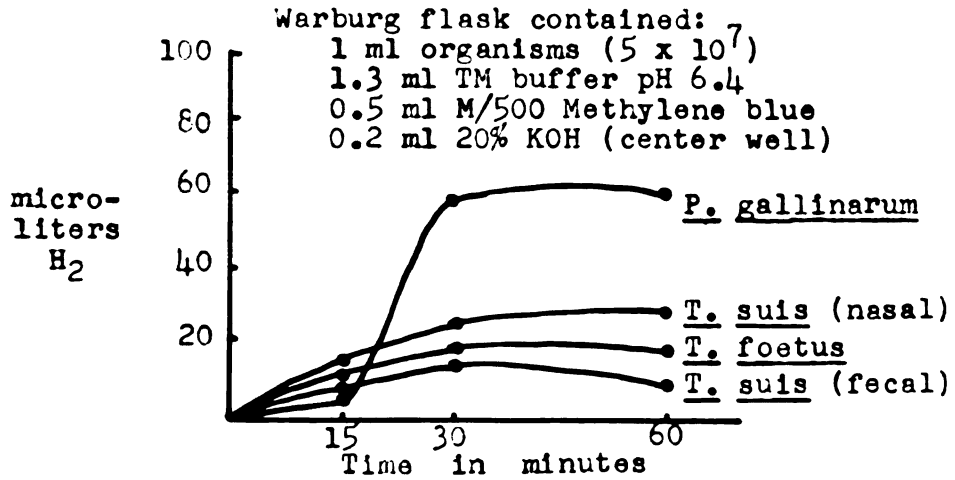


Figure 10. Hydrogenase Activity of Cell Suspensions of Four Trichomonads with Methylene Blue as Hydrogen Acceptor

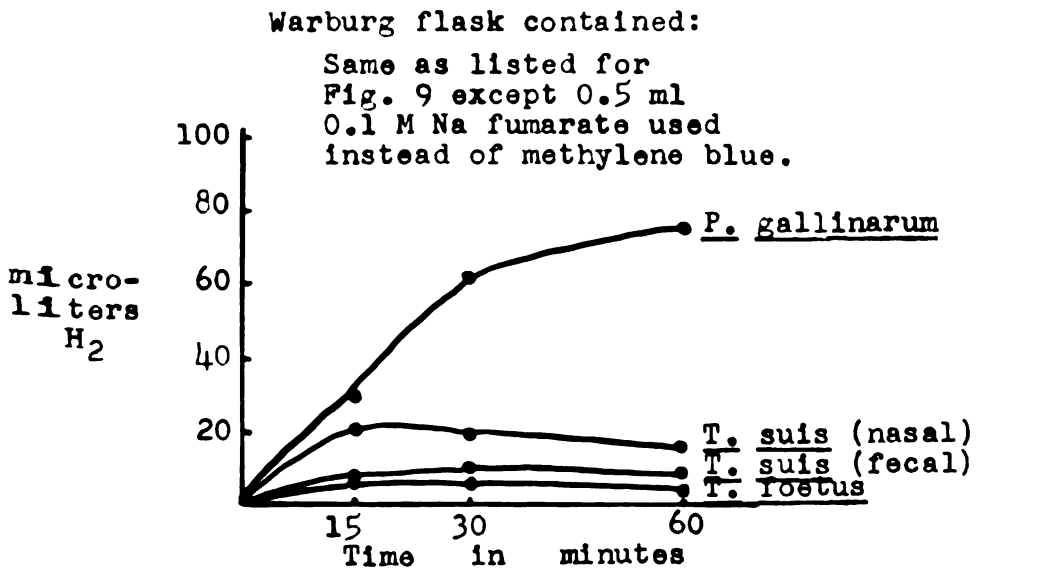


Figure 11. Hydrogenase Activity of Cell Suspensions of Four Trichomonads with Fumarate as Hydrogen Acceptor

was employed with cell-free sonic homogenates of the organisms. No increase in gas production in the presence of pyruvate over the endogenous rate was observed for any of the homogenates.

Enzymes of intermediary metabolism - Certain glycolytic and oxidative enzymes have been identified in cell-free preparations of T. foetus and T. vaginalis. Experiments were therefore designed to investigate the presence of these and other enzymes of carbohydrate metabolism in sonic homogenates of the four trichomonads in this study.

Evidence for strong hexokinase activity was demonstrated in all the homogenates by following CO_2 evolution from a bicarbonate buffer in the presence of adenosine triphosphate (ATP) and Mg^{++}. Figure 12 indicates the results of this experiment. This enzyme catalyzes the phosphorylation of glucose by ATP (Reaction 1).

(1) Glucose + ATP ⟶ Glucose-6-phosphate + ADP

This results in the liberation of one acid equivalent per mole of glucose phosphorylated, and this acid liberates CO_2 from the buffer. It can be seen that the nasal form is most active of the four.

(2) Glucose-1-phosphate ⟶ Glucose-6-phosphate

Evidence for phosphoglucomutase, the enzyme responsible for reaction (2), and therefore important in polysaccharide breakdown, was measured by incubating the homogenates with two micromoles glucose-1-phosphate

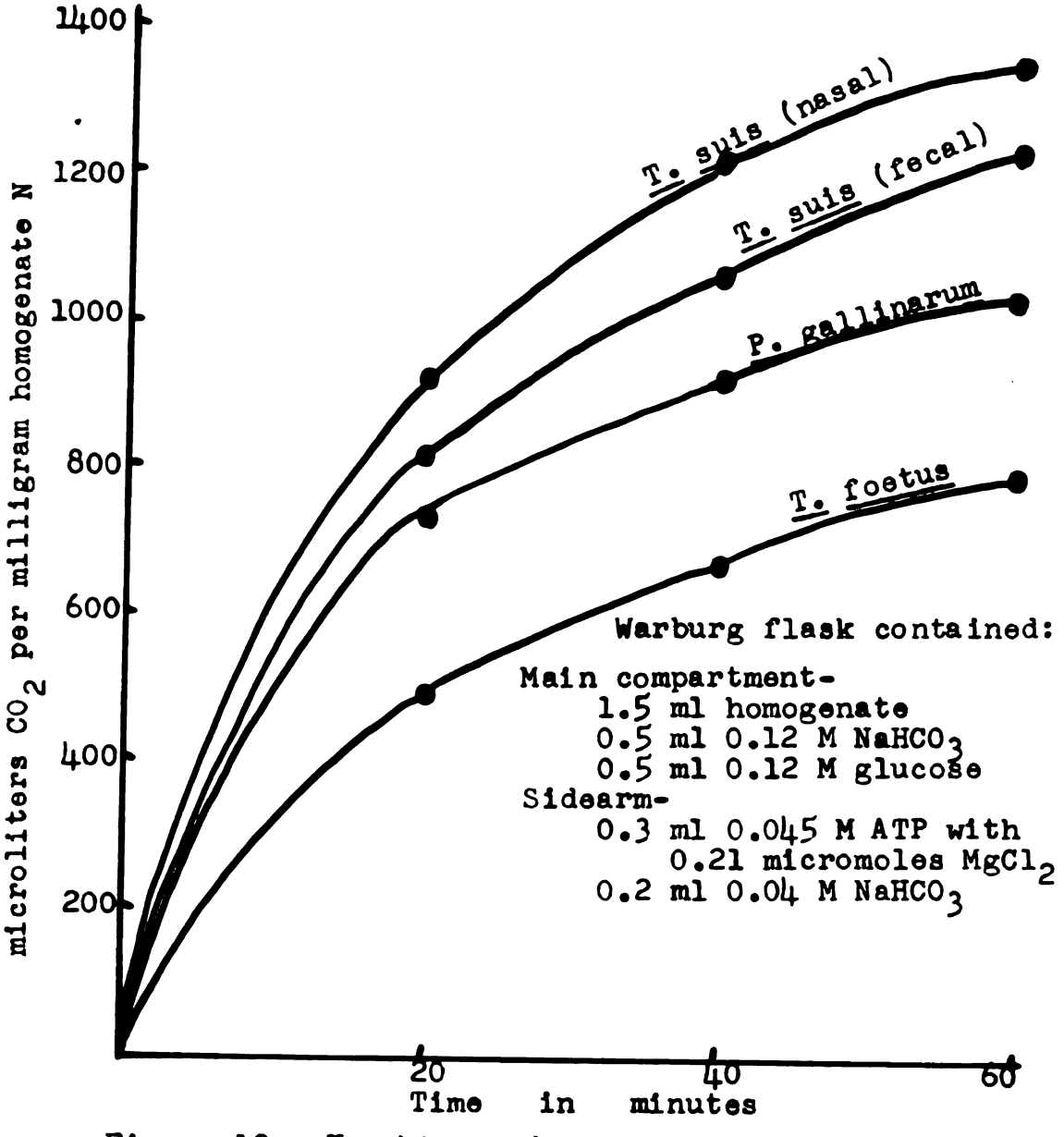


Figure 12. Hexokinase Activity of Trichomonad Sonic Homogenates.

(acid-labile phosphate) and measuring the decrease in acid-labile phosphate (glucose-6-phosphate is more stable to acid hydrolysis under the conditions of this experiment). Table 12 shows the results of these determinations.

TABLE 12

Phosphoglucomutase Activity of Trichomonad Sonic Homogenates

Organism	Homogenate (ml.)	Increase in Acid-Stable Phosphate (micromoles per mg cell nitrogen)
T. foetus	0.1	0.9
	0.3	1.8
P. gallinarum	0.1	0.4
	0.3	0.1
T. suis (fecal)	0.1	0.6
	0.3	1.3
T. suis (nasal)	0.1	0.2
	0.3	0.0

The reaction mixture contained 0.1 ml .006 M $MgSO_4$, 0.1 ml glucose-1-PO_4 (.02 M), 0.1 ml 0.1 M cysteine HCl, plus enzyme. Reaction stopped with 1 ml 5 N H_2SO_4. (pH during reaction = 7.5)

T. foetus and the fecal strain of T. suis showed definite activity but P. gallinarum and the nasal T. suis were inactive.

(3) glucose-6-phosphate ⟶ fructose-6-phosphate

Phosphohexoisomerase activity (Reaction 3) was extremely rapid in these homogenates, but a definite increase in fructose ester, as measured by the HCl-resorcinol method of Roe (1934), was seen in all the incubates. Figure 13 gives the data for these experiments.

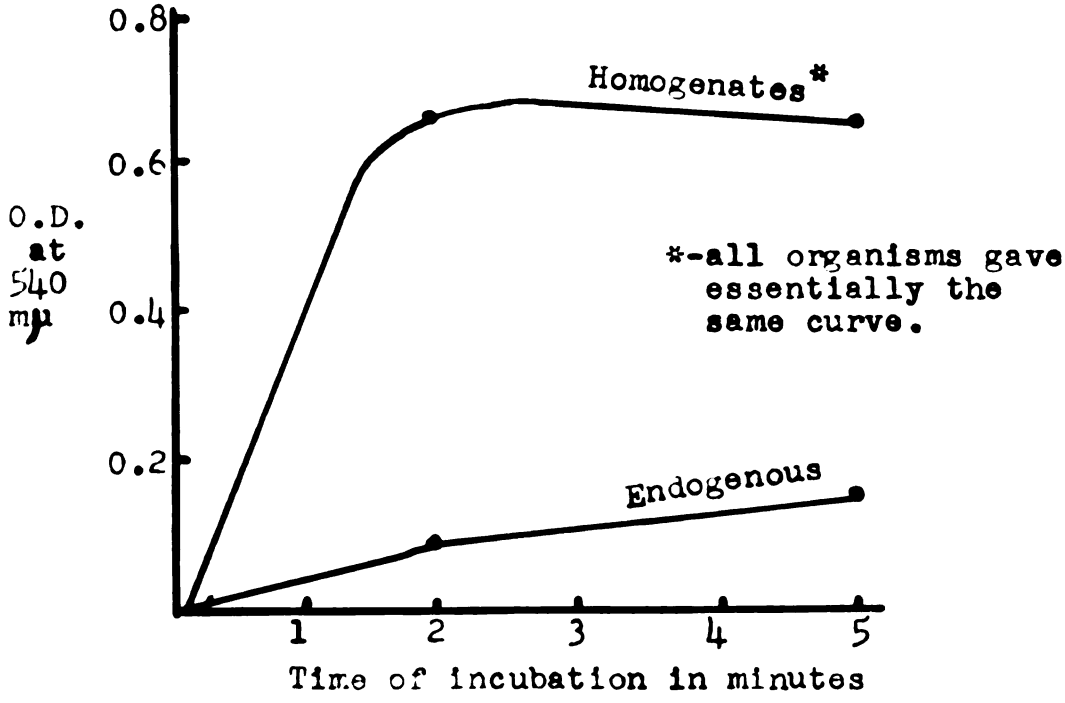


Figure 13. Phosphohexoisomerase Activity in Trichomonad Sonic Homogenates

The phosphorylation of fructose-6-phosphate by ATP, the next step in glycolysis (Reaction 4), is catalyzed by the enzyme phosphofructokinase.

(4) fructose-6-phosphate + ATP → fructose-1,6-diPO_4 + ADP

Evidence was obtained for presence of this enzyme in the homogenates of all the organisms using the same method as that for aldolase (Reaction 5) except that fructose-6-phosphate and ATP were used instead of hexose diphosphate.

(5) fructose-1,6-diPO_4 → dihydroxyacetone phosphate + 3-phosphoglyceraldehyde

The data are shown in Figures 14-17. Several attempts to demonstrate triosephosphate isomerase activity (Reaction 6) by omitting hydrazine from the digestion mixture for aldolase, on the assumption that isomerase would cause an increase in dihydroxyacetone phosphate thereby increasing the "chromogen", were unsuccessful.

(6) 3-phosphoglyceraldehyde ↔ dihydroxyacetone phosphate

In every case, a decrease in color resulted on omission of hydrazine. Baernstein and Rees (1952) and Baernstein (1953) were able to show isomerase activity in T. cruzi by this method, and Vandemark and Wood (1956) used it to show evidence of the enzyme in Microbacterium lacticum. It is interesting to note however, that Baernstein (1955) makes no mention of this in connection with later work on Trichomonas vaginalis.

Measurement of pyruvic acid formed on incubation of

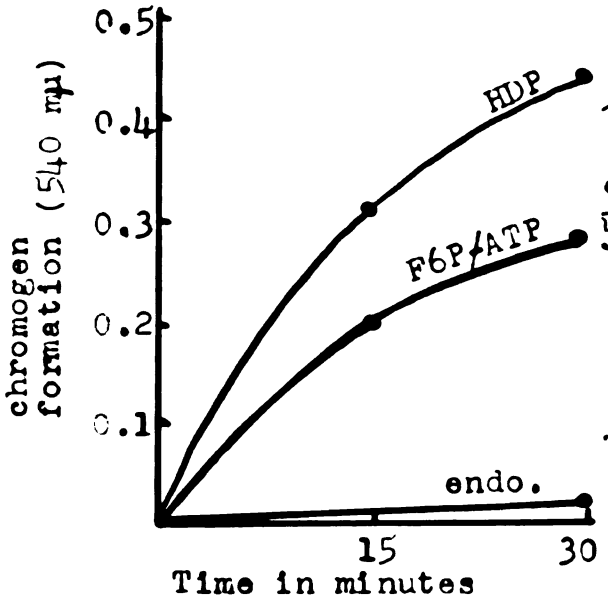


Figure 14.-T. foetus

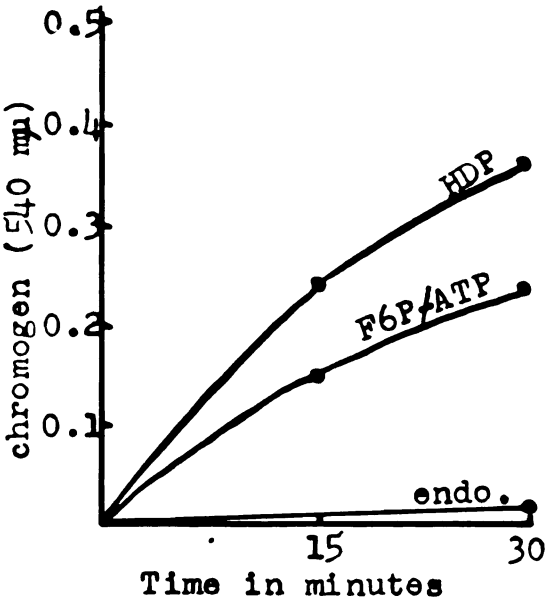


Figure 15.-P. gallinarum

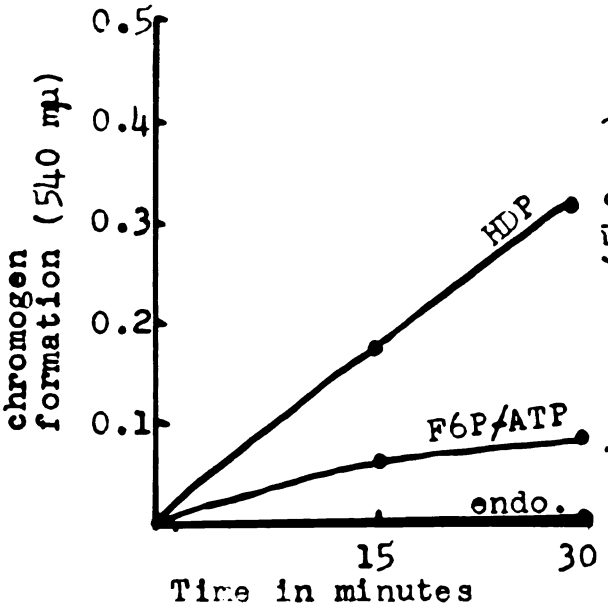


Figure 16.-T. suis (F)

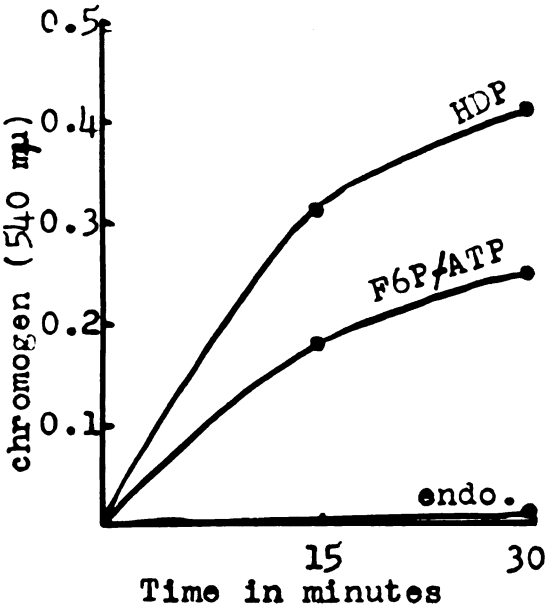


Figure 17.-T. suis (N)

Figures 14-17. Aldolase and Phosphofructokinase Activity of Trichomonad Sonic Homogenates.

homogenates with phosphoglyceric acid is an indication of the presence of phosphoglyceromutase and enolase (Reactions 7 and 8).

(7) 3-phosphoglyceric acid ⟶ 2-phosphoglyceric acid

(8) 2-phosphoglyceric acid ⟶ (enol)phosphopyruvic acid

The data given in Table 13 show that pyruvic acid increased in the tubes containing T. foetus and P. gallinarum homogenates, but not in the others.

TABLE 13

Phosphoglyceromutase and Enolase Activity in Sonic Homogenates of Four Trichomonads

Organism	Incubation time (min)	Micrograms pyruvate formed per mg homogenate N
T. foetus	10	5.4
	20	13.2
P. gallinarum	10	1.6
	20	5.6
T. suis (fecal)	10	0.0
	20	0.0
T. suis (nasal)	10	0.0
	20	0.0

The reaction mixture contained 2 ml 0.25 M glycylglycine buffer pH 7.4, 1 ml 0.05 M phosphoglyceric acid, 2 ml homogenate, and water to 6.5 ml. 1 ml aliquots removed at 10 and 20 minutes and added to 0.2 ml 40% TCA.

Additional studies were done to determine the existence in sonic homogenates of the trichomonads of malic, lactic, glycerol, glucose, and glucose-6-phosphate dehydrogenases, and fumarase.

No evidence was obtained for the presence of glycerol

dehydrogenase, glucose dehydrogenase, or fumarase, enzymes which are active in reactions (9), (10), and (11), respectively.

(9) glycerol + DPN → dihydroxyacetone + DPNH

(10) glucose + DPN → gluconolactone + DPNH → gluconate

(11) fumarate + H_2O → malate

An active malic dehydrogenase (Reaction 12) was found in homogenates of P. gallinarum and the nasal T. suis as shown in Figure 18, by following the increase in density at 340 millimicrons of a reaction mixture containing diphosphopyridine nucleotide (DPN).

(12) malate + DPN → oxalacetate + DPNH

In Thunberg experiments the same homogenates exhibited a reducing effect on methylene blue.

A weak, but definite, liberation of gas (CO_2) occurred when P. gallinarum homogenate (but not the others) was incubated with potassium malate and DPN (Figure 19). This corresponds to a "malic enzyme" activity (Reaction 13) seen in Lactobacillus arabinosus (Blanchard, et al., 1950).

(13) malate → lactate + CO_2

No work has been published on the possible importance of the hexose monophosphate shunt in trichomonad metabolism, although Sprince, et al. (1953) have indicated that ribonucleic acids are important for growth of these organisms. The first enzyme in this pathway, glucose-6-phosphate

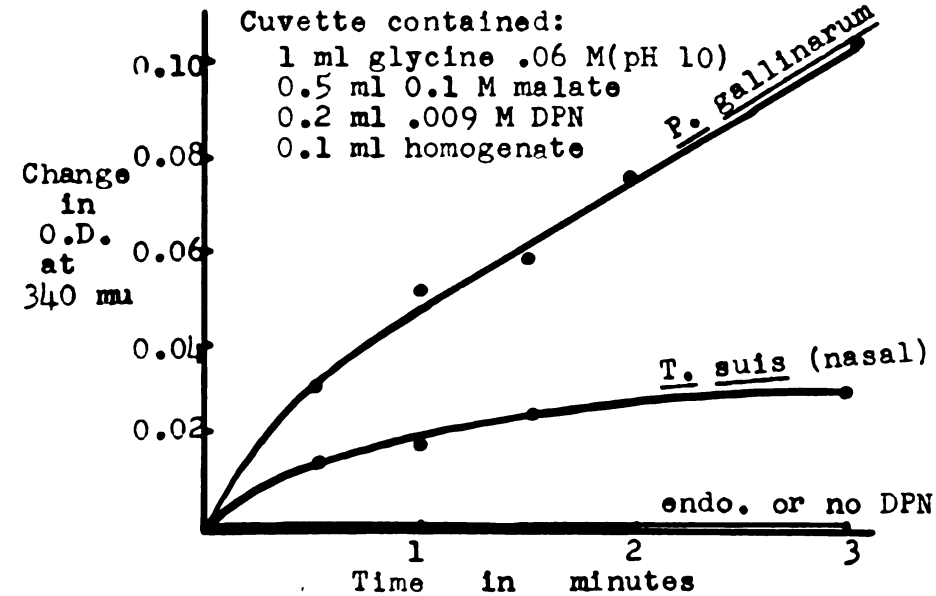


Figure 18. Malic Dehydrogenase Activity of Trichomonad Sonic Homogenates

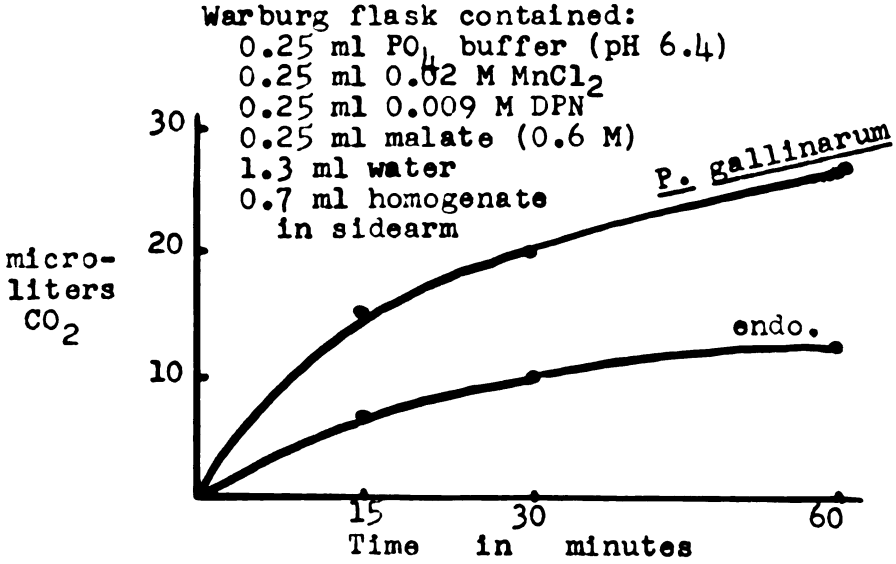


Figure 19. "Malic enzyme" activity in P. gallinarum Sonic Homogenate

dehydrogenase, which catalyzes reaction (14), has been reported from various micro-organisms.

(14) glucose-6-PO_4 + TPN⟶6-phosphogluconate + TPNH

In certain organisms it has been shown to be highly specific for one or the other of the pyridine nucleotides, while in others the enzyme appears to be active with either DPN or TPN.

Very active glucose-6-phosphate dehydrogenase activity, which was specific for TPN, was observed in three trichomonad homogenates. The nasal strain of T. suis showed weak activity. Figure 20 shows the results obtained when increase in density at 340 millimicrons was followed in a DU spectrophotometer.

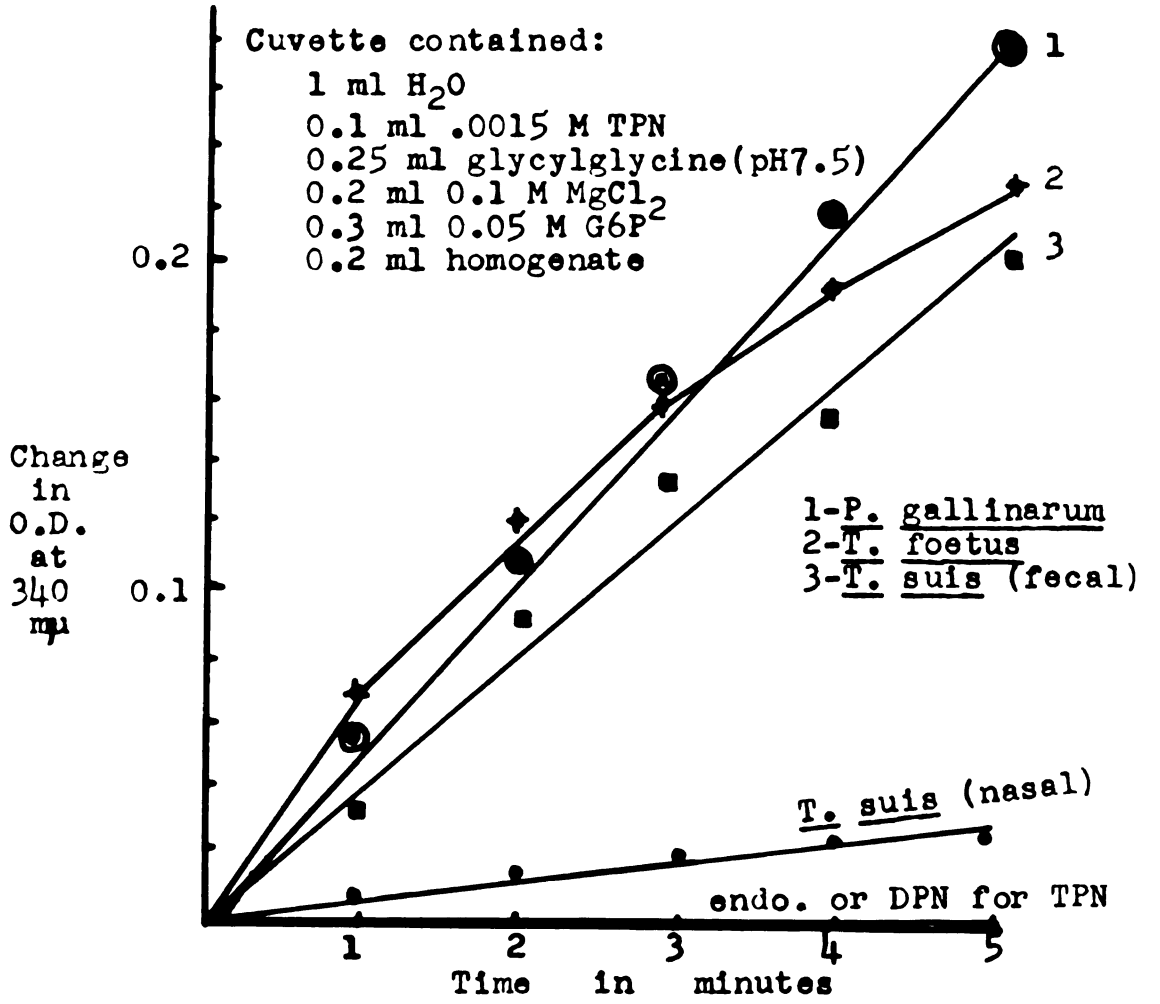


Figure 20. Glucose-6-phosphate Dehydrogenase Activity in Trichomonad Sonic Homogenates

DISCUSSION

The trichomonad flagellates appear to possess a rather strange type of carbohydrate metabolism. It consists, at least in part, of many of the reactions of the well-known Meyerhof-Embden pathway; but the organisms also have the ability to perform other reactions, not obviously connected with glycolysis. The tricarboxylic acid cycle does not appear to be present, although detailed studies with cell-free homogenates have not yet been reported.

In general, the organisms studied to date have behaved as facultative anaerobes. They gave evidence of preferring an environment of low oxygen tension, but still showed considerable activity under aerobic conditions. They metabolized carbohydrate substrates to a mixture of organic acids, notably succinic, lactic, acetic, and pyruvic, and also produced carbon dioxide and hydrogen. Evidence is also accumulating for the aerobic utilization of other substrates, such as pyruvate and malate, without evidence for the Krebs cycle.

In general outline, the organisms in the present study followed the pattern outlined above. However, certain differences were found to exist between the various species and, in some cases, differences from other work were noted.

The respiration of these organisms was affected by the age of the culture and the type and pH of the buffer

solution used in the experiments. Therefore, comparison of respiratory rates of certain organisms (e.g., Kupferberg, et al., 1953) without other experimental data is not strictly valid. The organisms in this study showed the greatest activity when less than 24 hours old, with a gradual decrease from 24-72 hours. This, and a fall in R.Q., has been indicated for other protozoa by Hutchens (1941)(Chilomonas), Baker and Baumberger (1941) (Tetrahymena), and Ryley (1955b)(Strigomonas). When the trichomonads were supplied with exogenous carbohydrate however there was no appreciable change in the R.Q. of organisms up to 48 hours old, showing that the flagellates were capable of actively using an external energy source even if their resting metabolic rate is slowed. It is still unknown, however, whether this respiration provides energy for growth.

The data on acid production showed that these flagellates produced more acid when harvested during the early and late periods of their growth, with a lag in between. Since acid production is usually evidence of an anaerobic metabolism, this result would indicate that in young cultures, and also as the organism ages, the glycolytic pathway is preferred.

The nasal form of T. suis was exceptional because of its constantly high rate of acid production. This was also reflected in the fact that a greater amount of

acid end products accumulated in cultures of this organism. In fact, this rather striking difference often made it difficult to work with cultures of the nasal strain since toxic pH levels were reached before satisfactory populations were attained. Additional evidence for increased glycolysis in the nasal form was offered by the observation of a more active hexokinase and the fact that utilization of all the sugars tested was greater than in the other three organisms tested.

Sugars utilized by T. foetus and the swine forms in the present study were essentially the same as reported by others. However, it is important to note that Ryley's (1955a) comment that strain differences apparently do exist in these protozoa can be confirmed by this work. The MSU strain of T. foetus resembled that used by Doran (1956b) more closely than either that of Ryley (loc. cit.) or Suzuoki and Suzuoki (1951a). All utilized glucose, fructose, mannose, and galactose, but Ryley reported that lactose was also oxidized by his strain, and the Japanese workers mentioned sucrose and maltose as stimulatory. Doran (loc. cit.) indicated that maltose was only slightly used, while the MSU organism used sucrose, but attacked maltose only slightly. The use of all sugars by the nasal T. suis, as was shown here, was also reported by Doran.

The production of hydrogen by these protozoa is of great interest from the standpoint of comparative bio-

chemistry. Suzuoki and Suzuoki (1955a) reported an active formic dehydrogenase in T. foetus, but no evidence for hydrogenase or formic hydrogenlyase. Ryley (1955a) was unable to detect any dehydrogenase activity. The organisms in this study all showed a strong formic dehydrogenase, but only a weak indication of hydrogenase. Formic hydrogenlyase was indicated only slightly by the adaptation experiment reported here. This evidence, however, together with the negative results of the "phosphoroclastic split" reaction suggests that here hydrogen is produced by a method more similar to Escherichia and Salmonella than Clostridium (i.e., like the facultative organisms rather than like the anaerobes). Nevertheless, the two forms of T. suis appeared to release hydrogen from pyruvate and not from formate. The phosphoroclastic enzyme is known to be very labile and, although every precaution was taken, it is possible that it became inactivated during preparation or storage of the sonic homogenates. This could explain the apparent discrepancy and more detailed work on this enzyme is indicated.

These organisms differ from other parasitic flagellates studied to date (e.g., Trypanosomidae) in being able to degrade carbohydrate past the pyruvate stage. Evidence has been available for many of the enzymes in the Meyerhof-Embden scheme for T. vaginalis and T. foetus.

In this work the presence of certain key enzymes in this scheme has been indicated for all the organisms. This confirms earlier work on T. foetus and provides similar information for the other organisms studied.

The inability to demonstrate triosephosphate isomerase activity by the methods involving omission of hydrazine from the digestion mixtures is interesting. This result might probably be explained by assuming that an active alpha-glycerophosphate dehydrogenase is present in all these organisms as has been reported for T. vaginalis. This enzyme would form alpha-glycerophosphate from the excess dihydroxyacetone phosphate obtained, and thus cause a reduction in color. This dehydrogenase acyivity was not tested in these organisms since a purified substrate was not available. However, since glycerol is not used by these organisms, the importance of this reaction is not clear.

The marked inhibitory effect of iodoacetate indicates that glycolysis normally proceeds past the triosephosphate stages; but it is also important to note that fluoride (an enolase inhibitor) was antagonistic (aerobically) to only T. foetus and T. gallinarum, the organisms which showed pyruvate formation from phosphoglyceric acid. This suggests that at least these latter two organisms produce pyruvate (and perhaps lactate) by conventional means under anaerobic conditions. The

stimulation of acid production by fluoride under anaerobic conditions could perhaps result from the inhibition of enolase and a coupled oxidation-reduction of two moles of triosephosphate to give alpha-glycerophosphate and 3-phosphoglyceric acid, as has been postulated by Ryley (1955b) for Strigomonas oncopelti.

Arsenate stimulation is compatible with the presence of a triosephosphate oxidizing system since addition of this ion increases the available esterifying material, and oxidation of the arseno-phosphoglyceraldehyde may not require arsenate acceptors, thus not limiting the rate of the reaction (Werkman and Schlenk, 1951). This has been observed by Speck and Evans (1945) in their studies on glycolysis in malaria parasites.

The pathway of formation of the organic acids formed as end products of trichomonad metabolism presents an interesting problem. In the absence of detailed information, speculation as to the possible schemes involved is the best that can be done.

The presence of enzyme systems active with malate is especially interesting. Malate can obviously arise from pyruvate or lactate by carbon dioxide fixation (the Wood-Werkman reaction). It could then be degraded through fumaric acid to succinic acid. (Failure in this work to demonstrate fumarase might be due to unrecognized experimental error; however, fumarate was shown to act

as a hydrogen acceptor, especially with P. gallinarum.) The efficiency of the organisms in fixing CO_2 would determine the small amount of pyruvic and lactic acids found as end products at any one time. It is important to note that Baernstein and Rees (1952) indicated a malate system in T. cruzi. They suggested that a linkage of this system to glutamic and aspartic acids through transaminase enzymes might prove to be more important than the glucose system in explaining respiratory activity.

Under anaerobic conditions pyruvate could form lactic and acetic acids, as well as CO_2, by the well-known Krebs dismutation (Reaction 15).

(15) 2 pyruvate ⟶ lactate + acetate + CO_2

Pyruvate could also, as indicated before, participate in a "phosphoreclastic" reaction giving rise to formate and acetyl phosphate ("active" C_2) and eventually to hydrogen and CO_2. The possibility of a 2-C_2 condensation to succinate or malate appears here.

Another interesting possibility is the probable presence of a hexose monophosphate shunt mechanism in these organisms, suggested by the demonstration of glucose-6-phosphate dehydrogenase. By this pathway triosephosphate and an "active" C_2 fragment are formed. A 2-C_2 condensation to succinate or malate as referred to earlier might be possible here. Also, Ochoa, et al. (1950) have shown that fixation of CO_2 by pyruvate in

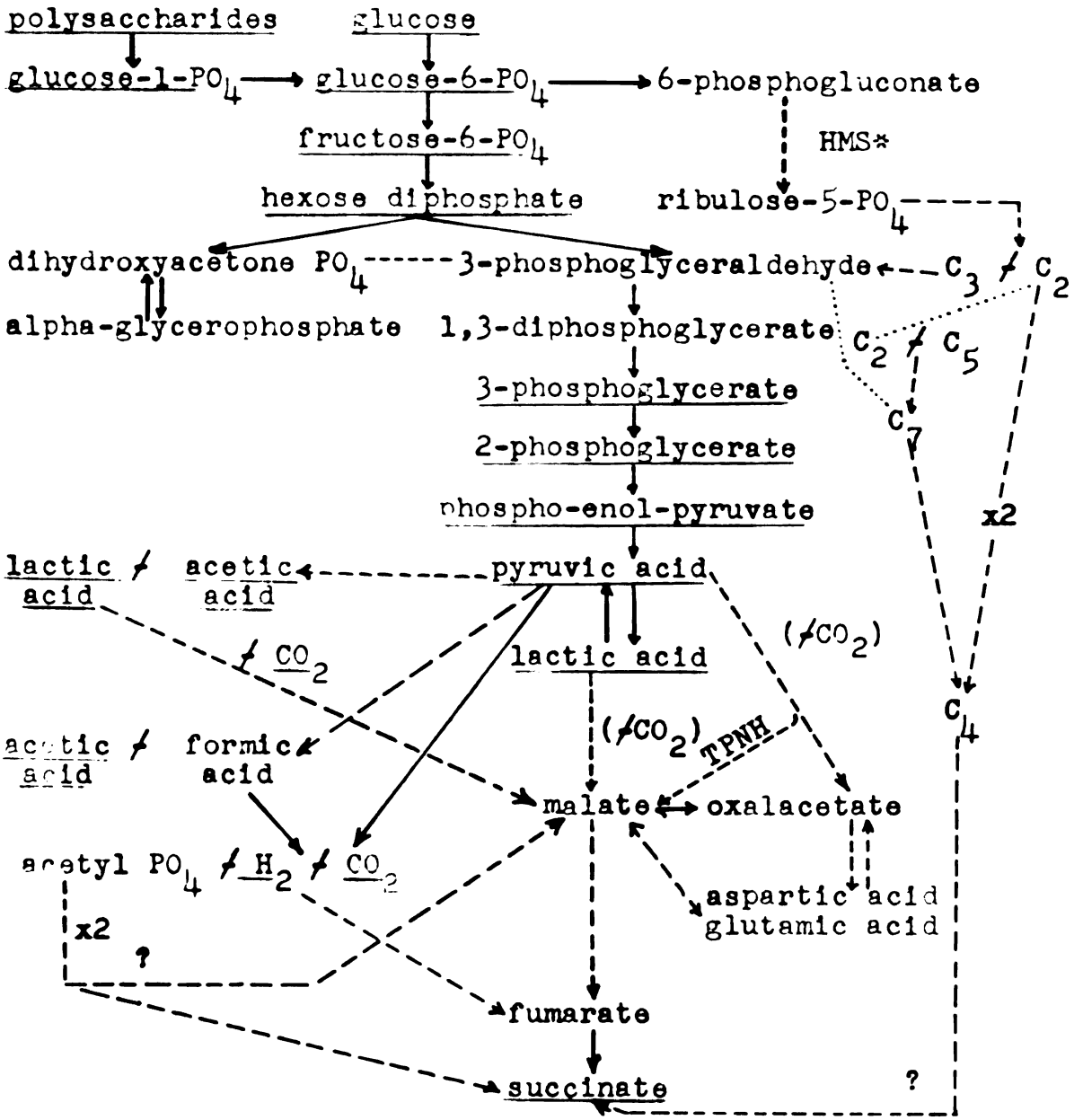
the presence of reduced TPN (from the glucose-6-phosphate dehydrogenase reaction) can give rise to malate.

Another hypothesis would involve the formation of ribulose-5-phosphate via the hexose monophosphate shunt followed by its splitting into triosephosphate plus a C_2 fragment which could condense with ribose forming sedoheptulose. This C_7 sugar could possibly split to another triosephosphate and a C_4 compound which might then proceed to succinate.

The identity of the terminal oxidizing systems of these organisms remains an unanswered question. Insensitivity of respiration to cyanide and azide, and the lack of evidence for cytochrome absorption bands in spectroscopic examinations, speak against this type of system. In all probability the flavin nucleotides are important in the trichomonads although much more work remains before details will be apparent.

The scheme shown in Figure 21 is an outline of the above discussion, showing the possibilities which exist in the breakdown of carbohydrates by trichomonads. It appears that detailed investigation of the reactions involving pyruvate and malate is indicated.

Besides attempting to investigate certain aspects of carbohydrate metabolism in these trichomonads, it was also a purpose of this work to discover, if possible, biochemical differences between the organisms employed,



*-HMS= hexose monophosphate shunt

Reactions which have been observed in trichomonads are indicated by solid lines. Those which are postulated are indicated by broken lines. The products which have been identified in any trichomonad are underlined in red.

Figure 21. Possible Metabolic Inter-relationships in the Carbohydrate Metabolism of Trichomonads

especially the two swine forms.

The apparently greater acid-producing capacity of the nasal T. suis has already been mentioned. Also, this organism appears to differ from its fecal counterpart by the fact that over 50% of its acid production can be accounted for as succinic acid. The fecal strain forms succinic as only 15% of its total acid, and also evidently produces a great deal of volatile acids (unaccounted for in the present analyses). With regard to this acid formation the nasal T. suis more closely resembled T. foetus than T. suis (fecal). This resemblance has been pointed out, on morphological grounds, by Buttrey (1956).

The respiration of both swine forms was more stimulated by exogenous carbohydrate than that of either T. foetus or P. gallinarum. Respiratory stimulation by disaccharides also differed, lactose and maltose being used only by the nasal form, and sucrose only by T. foetus.

Intracellular glycogen storage was considerably greater in T. suis (fecal) than in T. suis (nasal), suggesting that the nasal organism, more than the fecal form, required or preferred supplied, rather than formed, carbohydrate for energy.

Both swine organisms also differed from T. foetus and P. gallinarum by not utilizing lactate or formate for increased hydrogen production. This, and their weak response in the hydrogenlyase adaptation experiment,

would seem to indicate the presence of a "phosphoroclastic" system in these organisms. On the other hand, evidence for phosphoglyceric acid dissimilation to pyruvate was not demonstrated in homogenates of the swine organisms, but was found in the other two.

P. gallinarum showed considerably more malate activity than any of the others, a possible indication that this intermediate is more important in its metabolism.

SUMMARY

(1) T. foetus, P. gallinarum, and the fecal and nasal forms of T. suis were found to grow well in NIH Thioglycollate Broth (Case or Difco) with the addition of 1% sterile beef serum (Difco Beef Blood Serum, Dehydrated). Populations sufficient for manometric experiments were obtained in 30-40 hours if the original inoculum was reasonably large. Special preliminary cultures were used to insure this.

(2) During growth large amounts of acid were produced from glucose by all the organisms. The nasal T. suis however, was the most active in this regard. The pH at the time of maximum population was between 6.0 and 6.3 for all species and living organisms disappeared almost entirely at pH 5.2. These results are similar to data previously reported for other trichomonads.

(3) Succinic acid was the major acid produced by T. foetus, P. gallinarum, and the nasal T. suis, accounting for over 50% of the total acid formed in each case. T. suis (fecal) produced more lactic than succinic acid, but much of its acid is unaccounted for in these experiments. It is postulated that this might be acetic acid.

(4) The endogenous metabolic activity of the organisms was shown to vary with age at the time of harvest from culture, being greatest in young forms, and decreasing with advancing age. The composition of buffers was also shown to be important in the results obtained. With an external supply of carbohydrate the R.Q. of the organisms was constant for cultural ages of 12-48 hours.

(5) Glucose, mannose, fructose, and galactose were most active in stimulating respiratory activity. P. gallinarum, however, used fructose and galactose more slowly than the others. Disaccharides did not stimulate the respiration as well as did the monosaccharides. Maltose and lactose were used by the nasal form only, and sucrose by T. foetus alone. Intracellular glycogen synthesis was indicated with most of the sugars.

(6) Iodoacetate was the most potent inhibitor of the aerobic or anaerobic metabolism of all the species studied. Arsenate was stimulatory to all but P. gallinarum, and malonate failed to affect the respiration of any of the organisms. Hydrogen peroxide was not inhibitory since all the flagellates possessed strong catalase activity.

(7) Hydrogen production by these organisms decreased with cultural age; and it was stimulated by formate

in T. foetus and P. gallinarum, and by pyruvate in the swine forms. Presumptive evidence for a hydrogenlyase system (at least in T. foetus and P. gallinarum) was obtained. Formic dehydrogenase activity was marked in all the species.

(8) Hexokinase, phosphohexoisomerase, phosphofructokinase, aldolase, and glucose-6-phosphate dehydrogenase were demonstrated in sonic homogenates of each of the four organisms. Phosphoglucomutase activity was seen in preparations of T. foetus and the fecal T. suis. Production of pyruvic acid from phosphoglyceric acid, an indication of phosphpglyceromutase and enolase activities, was seen only in T. foetus and P. gallinarum, the only two which exhibited susceptibility to fluoride inhibition. T. suis (fecal) and P. gallinarum were found to possess malic dehydrogenase, and P. gallinarum also showed "malic enzyme" activity.

(9) Possible biochemical inter-relationships, based on available information for trichomonad metabolism, are discussed. It is felt that investigation of reactions involving malate and pyruvate will provide a key to understanding the complex life processes of these flagellates.

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SOME ASPECTS OF THE CARBOHYDRATE METABOLISM OF CERTAIN TRICHOMONADS

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AN ABSTRACT

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ABSTRACT

It was the purpose of this work to investigate the metabolic activities toward carbohydrates of four species of trichomonads, to relate the information gained to other flagellates which had been studied, and to discover, if possible, differences between the four species.

It was found that all species studied (*T. foetus*, *P. gallinarum*, and *T. suis* nasal and fecal strains) grew well in NIH Thioglycollate broth with the addition of 1% sterile beef serum. During growth in this medium the organisms produced various acid products from the glucose it contained. Succinic acid was the major acid produced by *T. foetus*, *P. gallinarum*, and the nasal *T. suis*, accounting for more than 50% of the total acid in each case. The fecal *T. suis* produced more lactic acid than succinic acid, but about 45% of the total acid was unaccounted for (probably volatile acid such as acetic). Pyruvic acid was found in small amounts in all cultures. The nasal form of *T. suis* produced much more total acid than the others. During growth the pH of the medium fell to about 5.2 at which point all organisms were dead. The pH at the time of maximum population was between 6.0 and 6.3. These findings were consistent with other work on *T. foetus* and *T. vaginalis*.

The metabolic activities of the organisms (oxygen uptake, CO_2 production, hydrogen evolution, anaerobic acid formation) varied with the age of the organisms. Respiratory activity was highest at about 12 hours, but varied considerably thereafter. Hydrogen was produced in greatest amount when the organisms

were about 12 hours old, showing a gradual decline thereafter. Acid production was high at 12 hours, fell between 20-30 hours, and rose again by 48 hours. With an external carbohydrate source the R.Q. of the organisms was consistent throughout growth.

Glucose, mannose, fructose, and galactose were most active in stimulating respiration. Disaccharides were slowly utilized. Intracellular glycogen synthesis occurred on incubation with most of the sugars. Iodoacetate and fluoride were the most active inhibitors of sugar utilization. Malonate was not effective. Cyanide and azide were ineffective also, as was H_2O_2 since all the organisms showed strong catalase activity.

Evidence was obtained for the presence in these organisms of hexokinase (greatest activity in the nasal form), phosphohexoisomerase, phosphofructokinase, aldolase, and glucose-6-phosphate dehydrogenase. Phosphoglucomutase was demonstrated in T. foetus and the fecal T. suis. T. foetus and P. gallinarum showed evidence of phosphoglyceromutase and enolase. T. suis (fecal) and P. gallinarum were found to possess malic dehydrogenase, and P. gallinarum gave evidence of "malic" enzyme activity. Formic dehydrogenase activity was marked in all forms, and presumptive evidence for a formic hydrogenlyase system was obtained (at least for T. foetus and P. gallinarum).

A discussion is presented indicating that investigations of those reactions which could involve pyruvate and malate (with a possible linkage with a hexose monophosphate shunt) might provide a key to a more complete understanding of trichomonad metabolism of carbohydrates.