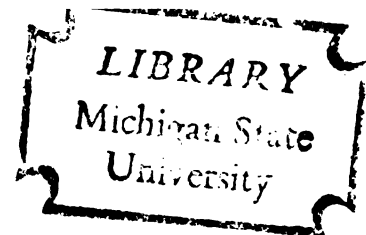


STUDIES ON THE GLUCOSE METABOLISM
OF TRITRICHOMONAS AUGUSTA

Thesis for the Degree of Ph. D.
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STUART H. WINSTON

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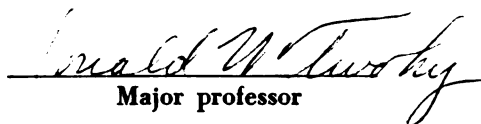


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STUDIES ON THE GLUCOSE METABOLISM
OF TRITRICHOMONAS AUGUSTA

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ABSTRACT

STUDIES ON THE GLUCOSE METABOLISM OF TRITRICHOMONAS AUGUSTA

by Stuart H. Winston

With glucose as an energy source, Tritrichomonas augusta produced lactic acid, acetic acid, succinic acid, CO_2 and H_2 from growth in a continuous culture system or under anaerobic conditions in a Warburg flask. The acids were extracted with ether, separated on a celite chromatographic column and titrated to determine the quantity of each acid. The gases were determined using a fermentation train with the continuous culture system or standard manometric techniques with the Warburg apparatus. Fermentation balances gave carbon recoveries of 89% from the continuous culture system and 95% from manometric studies. Oxidation-reduction balances were 0.81 and 0.85 respectively, showing a slight excess of reduced end product. The inherent errors of determining fermentation balances from organisms grown on undefined medium and under non-nutrient conditions with a glucose substrate were discussed.

Evidence was obtained for the occurrence of an Escherichia coli-type of phosphoroclastic split of pyruvate in Trit. augusta as the source of acetate, CO_2 and H_2 . The evidence consisted of the detection of acetyl phosphate and formate as intermediates plus a complete formic hydrogen-lyase enzyme system. The possibility of the formation of ATP via acetyl phosphate was discussed.

The detection of the malic enzyme, fumarase, and succinic dehydrogenase, the presence of malate and fumarate and the absence of citrate,

isocitrate, α -ketoglutarate and oxaloacetate in cell homogenates of Trit. augusta suggest the formation of succinate by a partial reversal of the Kreb's cycle. The malic enzyme resembled the enzyme system described for pig liver in its requirement for NADP as a co-factor. Fumarase was demonstrated for the first time in trichomonads by a complex coupling of the fumarase from cell extracts with added malic dehydrogenase and the use of two artificial co-factors for transfer and acceptance of electrons. The succinic dehydrogenase detected in Trit. augusta also seems to resemble the mammalian enzyme in that it is readily inhibited by malonate.

Trit. augusta has been shown to possess at least two pathways for the oxidation of NADH. One was via the lactic dehydrogenase system and the second was through the succinic acid-electron transport system. The latter system couples FAD reduction with NADH oxidation. Phosphorylation was associated with the above reaction resulting in nearly one mole of ATP formed for every NADH oxidized. The importance of this system in the overall energy metabolism of Trit. augusta was discussed.

**STUDIES ON THE GLUCOSE METABOLISM
OF TRITRICHOMONAS AUGUSTA**

By

Stuart H. Winston

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INTRODUCTION

Tritrichomonas augusta (Alexeieff) is a highly plastic pyriform protozoan ranging in size from 15 to 27 microns long by 13 to 15 microns wide. In addition to three anterior flagella, a fourth forms both the outer margin of the undulating membrane and the trailing flagellum. A rod-like costa follows the base of the undulating membrane, while a thick central axostyle extends the length of the body and protrudes posteriorly. The organism possess other structures commonly found in other animal cells such as the golgi apparatus (parabasal body), vacuoles and endoplasmic reticulum. Abnormal mitochondria-like structures lacking cristae have been reported in Trit. muris (1) and in Trichomonas criceti (7), but the actual presence of mitochondria in trichomonads has been questioned in recent investigations on Trit. fetus (47) and T. vaginalis (48).

Trit. augusta is a common commensal living in the colon of tadpoles, frogs and toads. The normal environment of this organism has a low oxygen tension and is rich in other microorganisms. Bacteria, unadsorbed food and intestinal secretions present in the lumen of the colon probably serve as food for this protozoan. Trichomonads can only be cultured in complex undefined media at low oxygen tensions. The optimum temperature and pH for growth approximate that of the host and the habitat in the host. Forms like Trit. augusta from cold blooded hosts grow in a temperature range of from 16 to 32 C and a pH range of 5.5 to 9.0 (22). The optimum generation time is from 6 to 8 hours.

The principle studies on the carbohydrate metabolism of trich-

omonads have been done on the pathogenic species. These organisms have been shown to utilize glucose, fructose, maltose and galactose and most of the common di- and polysaccharide sugars for growth or gas production. Pentoses and sugar alcohols do not generally support growth. There is no evidence of amino acids, lipids or other organic compounds, other than carbohydrates, serving as an energy source in the trichomonads studies to date. The nutritional requirements of trichomonads have been reviewed by Shorb (46).

Numerous investigators have determined one or more of the end products formed by these organisms as a result of anaerobic carbohydrate fermentation. Pyruvate, lactate, acetate, succinate, CO_2 and H_2 have each been detected by more than one of these investigators (23, 30, 37, 49 and 50). The scheme of formation of pyruvate and lactate have been well documented (2, 13, 23, 60), but the pathways for the production of succinate and acetate and the significance of these pathways to an electron transport system and energy production in the trichomonads has not been ascertained. Also, the type of mechanism involved in the evolution of H_2 and CO_2 has not been settled.

It is obvious that an understanding of the carbohydrate metabolism of the trichomonads lags far behind that of the bacteria. It was the purpose of this investigation to try and fill some of the gaps in the present knowledge of the glucose metabolism of Trit. augusta by:

(1) computing fermentation balances to provide a more accurate analysis of end products formed in relation to glucose utilized; (2) demonstrating the complete pathway by which succinate is formed; (3) determining the role and nature of the linkage of succinate production to the electron transport system; and (4) determining if formate is an intermediate in the production of CO_2 and H_2 .

LITERATURE REVIEW

The successful detection of the intermediates and enzymes of the glycolytic pathway fortified the theory of the existence of a conventional Embden-Meyerhof pathway in the trichomonads. Seven intermediates of the system were identified in T. vaginalis (61). They were: glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1-6-phosphate, 2-phosphoglycerate, 3-phosphoglycerate and phosphoenolpyruvate. All of the glycolytic enzymes have been demonstrated in T. vaginalis and most have been detected in other species of trichomonads (23, 40, 60). The only evidence for the existence of a hexose monophosphate pathway in these organisms is the detection of glucose-6-phosphate dehydrogenase in T. vaginalis (59) and in Trit. foetus, Trit. suis and Pentatrichomonas gallinarum (23).

End Products of Carbohydrate Metabolism: The end products from anaerobic growth on a carbohydrate substrate have been determined mostly for the pathogenic species of trichomonads. When cultivated for 48 hours in a complex medium containing glucose Trit. foetus was found to produce mainly H_2 with trace quantities of N_2 and methane gas. Of the total acid produced from glucose, 73 percent was succinic acid, and 22 percent was pyruvic and lactic acids (49, 50). Ninomiya and Suzuki showed that T. vaginalis could produce CO_2 and other unidentified gases from a medium in which either pyruvate or glucose served as the substrate (30). Kupferberg et al. were able to demonstrate only the evolution of CO_2 and the formation of lactate from carbohydrate medium, (20) but Read showed that glucose was metabolized to H_2 , CO_2 and unidentified acids by this same species (36). In later

work, Read was able to detect the anaerobic formation of CO_2 and H_2 by T. gallinae from glucose, fructose and galactose (37). Doran found that glucose, lactate and pyruvate stimulated gas production Trit. foetus and Trit. suis (11, 13). From 30 to 50 percent of the acid produced under anerobic conditions by these two species was lactic acid. He observed that nearly two thirds of the gas produced by these trichomonads from glucose was CO_2 , and that if no substrate was provided only one half of the gaseous end product was CO_2 . The addition of either lactate or pyruvate stimulated both gas production and oxygen uptake by these organisms. Other gases not adsorbed by KOH increased only slightly under these conditions. Tetratrichomonas buttreyi has been shown to produce similar end products from glucose (12). From 40 to 50 percent of the total acid was lactate. Gas production was also stimulated by glucose, pyruvate or lactate. Trit. augusta produces H_2 and CO_2 in equal or nearly equal amounts. The individual acids produced were not determined (53). Lindblom reported succinate to be the major acid produced by Trit. foetus, P. gallinarum and a nasal strain of Trit. suis in culture, but a fecal strain of Trit. suis produced more lactate than succinate (23). All of the organisms he studied formed CO_2 and H_2 .

Studies on Gas and Lactate Formation: The metabolic pathways for the production of most of the end products formed by the trichomonads have either to be discovered or to be verified. The presence of a glycolytic pathway in the trichomonads accounts for the formation of pyruvate. Lactate is produced from pyruvate by a conventional pathway involving reduced NAD and lactic dehydrogenase (2, 13). The scheme for the formation of H_2 and CO_2 from pyruvate is disputed. Lindblom

reported an adaptive type of formic hydrogenlyase system in Trit. foetus, P. gallinarum and in two strains of Trit. suis (23). He demonstrated an active formic dehydrogenase, but showed only a weak hydrogenase activity. Ryley on the other hand, thought it was likely that Trit. foetus possessed a phosphoroclastic system similar to that of the Clostridia (40). He based this assumption on his observations that Trit. foetus did not produce formate and could not use formate as a substrate.

Evidence for a Kreb's Cycle: A complete Kreb's cycle seems to be lacking in the trichomonads. The only enzymes from this cycle which have been demonstrated by direct methods are malic dehydrogenase in I. vaginalis, P. gallinarum and Trit. suis and succinic dehydrogenase in I. vaginalis (3, 23, 43). Read speculated on the presence of aconitase, isocitrate dehydrogenase and α -ketoglutaric dehydrogenase in I. gallinae based on the finding that the protozoan could oxidize a number of the intermediates of the citric acid cycle (37). There is no direct evidence for the presence of these enzymes. Neither the condensing enzyme nor fumarase have been detected by indirect or direct methods as this time (23, 61).

Kunitake et al. attempted to demonstrate the presence of the Kreb's cycle in I. vaginalis using glucose- $U-C^{14}$ or succinate-2,3- C^{14} as substrates. They were unable to isolate labeled Kreb's cycle intermediates from cells grown with either substrate (21). However, labeled CO_2 and amino acids were detected. These investigators stated that the pattern of labeling in the amino acids suggested the presence of a citric acid cycle in I. vaginalis. These investigators were unable to find any activity of these Kreb's cycle enzymes in their cell homogenates.

If some sort of di- or tricarboxylic acid pathway does exist in these organisms, there must be a mechanism for the formation of a four carbon intermediate. Since the condensing enzyme has not been detected, the trichomonads must use some other method for tri- and dicarboxylic acid production. In their first investigation, Wellerson et al. found labeled CO_2 was incorporated into the carboxyl group of lactate by I. vaginalis (58). Since it was not incorporated into malate or succinate, they believed CO_2 fixation onto pyruvate by the malic enzyme did not occur. Later, these same investigators demonstrated a very active NADP-linked malic enzyme in this trichomonad, suggesting a means for I. vaginalis to form malate from CO_2 and pyruvate (60). This type of enzyme was also found in Trit. fetus (25). Lindblom was able to show a weak reversal of the malic enzyme reaction in P. gallinarum by incubating trichomonad homogenates with malate and NAD and measuring the CO_2 evolved (23).

Studies on Terminal Respiration: Investigations into the terminal respiration of the trichomonads have led to many speculations. Several authors have failed to demonstrate the presence of cytochromes in these protozoan (40, 58). Only Suzuki and Suzuki reported a cytochrome b resembling the mammalian type in Trit. fetus (50). Whether or not this cytochrome really exists has been questioned. It is not unusual to find anaerobic organisms lacking a cytochrome system.

The most common electron carriers in anaerobes are the flavoproteins. Riboflavin and flavin mononucleotide have been isolated from I. vaginalis (58), as has flavin protein oxidase (3) and NADH oxidase (26, 58). The premises for the presence of an electron transport system in the

trichomonads was summarized by Baernstein (4): "Trichomonads are essentially anaerobic and therefore depend on coupled reactions mediated by pyridino and flavoproteins resulting in the production of reduced compounds. They depend on conventional glycolysis supplemented with other systems linked to NADP and to succinate to furnish electron donors. Electron transport in anaerobic metabolism is limited to dehydrogenase couplings and to the excretion of the reduced compounds. In some strains anaerobic production of hydrogen supplements the usual mechanism of electron transport."

Inhibitor Studies on the Kreb's Cycle and Terminal Respiration:

Common inhibitors of the citric acid cycle have been tested both on isolated enzyme systems and whole cells of trichomonads. Both beta-phosphoenolpyruvate and arsenoacetate were effective inhibitors of succinic dehydrogenase activity in cell extracts of T. vaginalis (43). Neither inhibitor has any effect on the succinic dehydrogenase from mammalian cells. Malonate a potent inhibitor of mammalian succinic dehydrogenase had no effect on oxygen uptake by the four species of trichomonads studied by Lindblom (23). Malic dehydrogenase from T. vaginalis was inhibited completely by p-mercuribenzoate but not by any of the common SH-inhibitors (30). Neither cyanide or carbon monoxide had any appreciable effect on the acid production of T. vaginalis (20) or on the oxygen uptake of Trit. fetus (10) and T. vaginalis (38). This suggests that terminal respiration in the trichomonads is not through the usual cytochrome system.

Variability in the Metabolism of Trichomonads: It is apparent that all of the species of Trichomonas so far studied can anaerobically metabolize the hexoses to CO_2 , H_2 and various organic acids. The

relative amounts of each end product depend on the species studied and on cultural conditions. Unfortunately, the wide variation in cultural conditions often makes it difficult to compare the work of different investigators. Read and Rothman (38) have called attention to variations in the Q_{acid} and Q_{gas} throughout the growth cycle of T. vaginalis. Q_{acid} and Q_{gas} also varied with the strain of the species being investigated. The variations were shown to depend upon the quality of the medium, age of the inoculum and the cultural history of the cells. Their conclusions were substantiated by Lindblom with Trit. foetus, Trit. suis and P. gallinarum (23). His work showed that oxygen uptake, CO_2 and H_2 evolution and anaerobic acid formation varied with the age of a culture. Gas production was highest at 12 hours of growth but varied considerably thereafter. Acid formation was highest at 48 hours.

Due to the above reported variations, the cells employed in this study were grown under constant conditions in a simple external control continuous culture apparatus (16). With this system a constant population of trichomonads could be maintained over long periods of time. Since this population would be in a steady state the variation in its metabolism from day to day would be expected to be at a minimum, giving a more reliable picture of the glucose metabolism of Trit. augusta. It is this type of stability which appears to be lacking in previous studies on the trichomonads.

METHODS AND MATERIALS

Culture Methods: A clone (5B) of the parent strain T-12 of Tritrichomonas augusta isolated from Rana catesbiana was used in this study. Stock cultures were maintained by serial passage in 16 X 125 mm screw cap test tubes containing 10 ml of Diamond's medium (8) without agar. The medium was adjusted to pH 6.6 to 6.8 with phosphate buffer and the cultures incubated at room temperature or at 29 C.

The continuous culture apparatus used in this study (52) consisted of: 1) a 1 l. reservoir flask containing fresh medium, 2) a modified 500 ml. tissue culture spinner flask (Belco Glass Inc. Vineland, N.J.) which served as a growth flask, 3) a magnetic stirrer to rotate the impeller in the growth chamber, 4) an overflow flask, and 5) a multispeed transmission pump (Harvard Apparatus Co., Dover, Mass.) model # 600-000 which was employed to force medium from the reservoir flask into the growth chamber.

A stock culture in the log phase of growth (approximately 48 hours old) was used to inoculate the growth flask of the continuous culture apparatus. When full, the growth flask contained 340 ml of Diamond's medium modified by the addition of 10% 0.11M Na_2HPO_4 (pH 6.6) and by the substitution of 2.0mM concentration of glucose for maltose. All of the above procedures of preparation, assembly and inoculation of the apparatus were carried out using sterile techniques.

To insure anaerobic conditions for growth the entire apparatus was flushed with nitrogen. Oxygen was removed from the nitrogen by passage through copper turnings at 500 C and carbon dioxide by

bubbling through 1 N NaOH. Trichomonad growth was limited by the low concentration of glucose used in the modified Diamond's medium. After inoculation the organisms were allowed to grow undisturbed for three days at 29 C. Then the impeller was started to give a homogeneous suspension of organisms in the medium and the pump turned on to force fresh medium into the system. In this study a flow rate (f) of 5.4 ml/hr. was used, providing a dilution rate (D) of 0.0158. D was determined from $D=f/v$ where v= the flask volume (340 ml). It represents the number of complete volume changes per hour. The mean residence time of an organism in the growth flask is equal to $1/D$ or 63.3 hours (16). The generation time (g) in hours calculated by the formula $g=\ln 2/D$, was 43.9 hours (14).

Population Determination: Steady state conditions were assumed to exist when the trichomonad population showed no consistent increase or decrease (16). Duplicate samples for population counts were withdrawn from the growth flask daily in a sterile 1 ml syringe fitted with a three inch 22 gauge needle which was inserted through a rubber stopper in one sidearm. Each sample was killed and preserved with 1 ml of 2% formalin diluted with 80% (v/v) Kreb's Ringers phosphate (KRP) and stored not more than 24 hours prior to counting. Samples were then diluted to 20 ml with 80% KRP and the cell population counted with a Model-A Coulter Counter (Coulter Electronics, Chicago, Ill.) equipped with a 100u diameter aperture. The instrument was set at an aperture current of 2 and a threshold value of 15. All counts were corrected for coincidental passage of more than one organism at a time through the aperture (28).

Harvest of Cells and Preparation of Homogenates: Approximately

240 ml of medium were decanted from the growth chamber of the continuous culture apparatus into the recently drained overflow flask. The medium was then flushed from this flask with nitrogen, collected in conical glass screw cap centrifuge tubes and centrifuged for 20 minutes at 880 X g. The supernatant was decanted and the cells were suspended in a small volume of 80% KRP with 0.1% cysteine, adjusted to pH 6.8 with 1N NaOH. The organisms were pooled and the process of suspension, centrifugation and decantation repeated until the cells were washed three times in cold 80% KRP-cysteine. The cells at this stage of processing were employed in experiments requiring whole cells, except for organisms used for the determination of cell nitrogen and glycogen. Cells used for these studies were washed in cold 80% KRP without cysteine.

For those experiments that required cell homogenates the following additional procedure was used to rupture the trichomonads. Cells from the final pellet were suspended in 3 ml of 0.02M phosphate buffer containing 0.1% cysteine (pH 6.8), placed into a Thomas model B10083 tissue grinder, immersed in an ice bath and homogenized at 1000 rpm for seven minutes. The homogenate was then dialyzed for 24 hours at 4 C against the same phosphate buffer with cysteine. After removal from the dialysis tubing, the homogenate was centrifuged at 880 X g for five minutes and the supernatant decanted and stored at -12 C for use the same day.

Determination of Gas Production: The CO_2 and H_2 produced by Trit. augusta were measured for short intervals with non-growing cells or over 24 hour periods of growth in the continuous culture system incorporated to be fermentation train described by Neish (29). In

the latter system O_2 and CO_2 -free nitrogen was flushed continuously through the apparatus. The metabolic CO_2 in the gas was first trapped in Ascarite. The H_2 was converted into water by passage through copper oxide at 550 C and adsorbed in calcium chloride. The Ascarite and calcium chloride traps were weighted before and after each experiment to determine the amount of gas absorbed.

Manometric experiments were conducted at 30 C in a Precision Scientific 20 unit Warburg respirometer with a shaking rate of 120/minute according to standard procedures (54). All experiments were conducted using an O_2 and CO_2 free nitrogen atmosphere.

Carbon dioxide retained in the Kreb's phosphate buffer system employed for the manometrically determined carbon balance was expelled by adding 3N H_2SO_4 from a sidearm at the end of the experiment (54). The H_2SO_4 was added to the contents of a control flask at the start of the experiment to determine the initial CO_2 in the buffer. Hydrogen was measured in flasks containing 20% KOH in the center well to absorb the CO_2 (54).

Determination of Organic Acids: Organic acids were separated from cells and medium using the method for celite partition chromatography described by Swim and Krampitz (51) and modified by Seaman (44). Eighteen ml samples of either medium or cell homogenates were first extracted with ether in a reflux condenser described by Melish (29) according to the method outlined by Seaman (44). The concentrated organic acid sample to be chromatographed was combined with 1 gram of celite (Johns-Mansville Filter Aid) in a mortar with a pestle and added to the top of a previously prepared celite column (45). A sequence of chloroform and chloroform-butanol mixtures described by Swim and Krampitz (51) were passed through the column to separate the

acids in the ether extracted sample. A positive pressure of N_2 was applied to the column to achieve a flow rate of 3-4 ml/min. Successive 5 ml fractions, regulated by the drop count, were collected with the aid of a fraction collector. Each fraction collected was titrated with 0.015 M alcoholic KOH until phenol red indicator gave a stable red color. The quantity of acid present in a given sample was determined from the volume of alkali required to neutralize each fraction in a given peak (44). The volumes of KOH were plotted for successive samples and the sum of the volumes for each peak converted to equivalents of organic acid. Samples of fresh medium were analyzed prior to their use in the growth chamber and the initial concentrations of any of the measured organic acids subtracted from the final values. Mixtures of standard organic acids were passed through the column to check on the positions of peaks and on the efficiency of the method.

Determination of Glucose, Glycogen and Nitrogen: The yeast extract and serum in the medium contained natural sugars in addition to the glucose component added. Prior to the determination of the glucose content of the medium, the sample was passed through a short anion-cation exchange resin column (4X5 cm). The resin was that normally used in a demineralizer (Deminite L 10 Resin, Crystal Research Labs., Hartford, Conn.). The column was washed with sufficient water to double the samples volume. The resin removed unknown non-carbohydrate reactants which caused a non-specific color reaction with anthrone reagent. Glucose was determined by Roe's (39) anthrone method.

For glycogen and nitrogen determinations the cells were removed from the growth chamber and freed of medium by the method previously

described for the harvest of intact cells. Glycogen was extracted from approximately 8×10^6 washed cells by the method of Good et al. (15) and assayed by Roe's anthrone method (39). Standards containing a known amount of glycogen were included in each group of analyses. Cell nitrogen was determined by the Nesslerization procedure described by Umbreit using a Bausch and Lomb Spectronic 20 spectrophotometer (54).

Fermentation balances based on substrate utilized and gas and acid end product analyses were calculated according to the method outlined by Wood (63). Analyses of fresh and used medium showed that pyruvate was utilized by Trit. augusta from the Diamond's medium. This pyruvate was considered as a substrate along with glucose in calculations of the fermentation balance.

Enzyme Assays: The general procedures employed in the spectrophotometric assays of malic enzyme (31), succinic dehydrogenase (5) lactic dehydrogenase (19), pyruvate carboxylase and phosphoenolpyruvate carboxylase (56) activity have been described in detail in Methods in Enzymology, vol 1. 1955. Other determinations of enzyme activity required modifications of described techniques.

The enzymes of the formic hydrogenlyase system were determined manometrically using the method of Peck, Jr. and Gest (33, 34). The components of each assay accompany the appropriate figures depicting the results.

The following coupled reactions were involved in the determination of fumarase activity in Trit. augusta:



- (3) $\text{NADH} + \text{H}^+ + \text{Phenazine Methosulphate (PMS)} \text{ ----- reduced PMS} + \text{NAD};$
 (4) $\text{reduced PMS} + \text{Triphenylterazolium Chloride (TPT)} \text{ ----- reduced TPT} + \text{PMS}.$

The fumarase present in the cell homogenate converted the fumarate to malate. Commercial malic dehydrogenase was added to catalyze the formation of oxaloacetate and reduced NAD. Phenazine methosulphate acted as an electron acceptor from $\text{NADH} + \text{H}^+$. It in turn reduced the indicator, triphenyltetrazolium chloride, a reaction which was followed spectrophotometrically at a wavelength of 650 mμ. To check the specificity of the enzyme in the cell extract, fumarate was omitted from the control assay.

The assay for a coupled electron-high energy phosphate transfer (EEPT) system was carried out in the cuvette of a model D. U. Beckman Spectrophotometer. To cell homogenate, which was dialyzed against N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) buffer, was added adenosine diphosphate (ADP), flavin adenine dinucleotide (FAD), phosphate buffer (pH 6.8) and reduced NAD as a source of electrons. The criteria of activity for the system was the measurement, at a wavelength of 340 mμ of the oxidation of the reduced NAD. One control was used for each component, i.e. with each component deleted from the system. TES buffer was used in place of phosphate buffer to determine if inorganic phosphate was essential for the activity of the transport system.

In spectrophotometric assays, rate measurements were conducted at ambient temperatures between 23 C and 26 C. Substrate or enzyme was added at zero time and the components mixed by inverting the covered cuvette a few times. Appropriate blanks were used to zero in the

spectrophotometer prior to each experiment.

Determination of the Effect of Malonate on Succinate Production:

In order to determine if the enzyme responsible for succinate production in Trit. augusta was susceptible to malonate inhibition the following experiment was performed. Two Thunburg tubes were prepared to contain final concentrations of 0.002M glucose, 3×10^6 cells and 0.01M malonate in 0.1M phosphate buffer (pH 6.8) to give a total volume of 10 ml. Control tubes contained the above materials minus malonate. All of the tubes were alternately placed under vacuum and flushed with O_2 free N_2 three times. The reaction mixtures were then incubated in a 30 C water bath for one hour. After removal from the bath the contents of the tubes were centrifuged at 880 X g for 10 minutes to remove the cells. The supernatant was then ether extracted (44) and placed on a celite chromatography column (51). The amounts of fumarate and succinate in both the control and experimental mixtures were determined as described earlier (44).

Determinations of Co-factors and Other Intermediates of Metabolism:

To determine FAD and riboflavin, 10 lambda of a 0.002M solution of each was spotted on Whatman # 4 filter paper strips (38X34 mm). Eighty lambda of cell extract was then spotted on a third strip of the same dimensions. An end of each strip was immersed in a solvent composed of 160 grams of phenol, 30 ml of butanol and 100 ml of water for 5 hours at 20 C (9). After the strips were air dried they were checked for the fluorescence of the flavins using a model R-51 Mineralite (Ultraviolet Products Inc., San Gabriel, Calif.). The Rf values were determined for these spots.

Adenosine triphosphate (ATP) was chromatographed on paper according

to the method described by Pabst Laboratories (32). An 80 lambda aliquot of sample, from the assay mixture used for determining the EEPT system was spotted on a 21X16½ inch sheet of Whatman # 1 filter paper. The control on the ATP chromatograph consisted of the above mixture minus reduced NAD. The filter paper was immersed in a solvent composed of 100 ml of 0.1M phosphate buffer at pH 6.8, 60 grams of ammonium sulphate and 2 ml n-propanol. After developing for 16 hours in an ascending position, the sheet of paper was air dried, the spots located by observing the visual quenching of ultraviolet light by ATP under a Mineralite lamp and the Rf values calculated. The spots were cut out of the chromatogram and placed in vials which contained 3 ml of 0.1N HCL. The vials were placed in a 37 C incubator overnight. The eluted nucleotides were then pipetted into 3 ml quartz cuvettes and their absorbancy at a wave length of 257 mu determined in a model D. U. Beckman Spectrophotometer. The concentration of ATP present in each sample was calculated using an extinction coefficient of 14.7×10^{-3} for ATP at 257 mu and pH 2.0.

The amount of NADH oxidized was determined for the EEPT reaction by measuring the change in optical density at 340 mu for one minute. The optical density value obtained was then incorporated into the following formula: $\underline{A_m} = \underline{A_s}/\underline{C}$, where, $\underline{A_m}$ equals the molar absorbancy index (extinction coefficient), $\underline{A_s}$ equals the absorbancy and $\underline{C}$ equals concentration. Using the $\underline{A_m}$ value of 6.22×10^{-3} the concentration of reduced NAD oxidized was determined from the spectrophotometric reading. The ratio of ATP formed per NADH oxidized was then calculated.

Acetyl phosphate was analyzed by the method of Lipmann and Tuttle (24). Samples for the assay were obtained from Warburg vessels

which contained the following: 4M hydroxylamine -HCl, 3.5M sodium hydroxide, 0.1M acetate buffer (pH 5.4), dialyzed cell homogenate, and 0.05M sodium pyruvate as a substrate. The atmosphere in the flasks was 100% N₂ and the reaction was carried out for one hour at 30 C.

Materials: The common organic and inorganic compounds used were of highest grade of purity available commercially. The other chemicals were obtained from the following sources: benzyl and methyl viologen from Mann Research Laboratories, New York, N. Y.; malic dehydrogenase, oxidized and reduced NAD and ADP from Sigma Chemical Co., St. Louis, Mo.; reduced nicotinamide adenine dinucleotide phosphate (NADPH), FAD and riboflavin from Calbiochem, Los Angeles, Calif.; and ATP from the Pabst Brewing Co., Chicago, Ill. The TES buffer was obtained through the kindness of Dr. Norman E. Good, Department of Botany, Michigan State University, East Lansing, Michigan.

RESULTS

1. The Glucose Fermentation Balance of Trit. augusta.

A. Continuous Culture System: A typical fermentation balance from the use of the continuous culture apparatus was summarized in table 1 (part 1). The mean trichomonad population was 1.24×10^6 cells per ml (0.036 mg N/ml) at the time a steady state was reached. The gases produced by the organisms were collected over a 24 hour period. Organic acid, glucose, cell nitrogen and glycogen determinations were made on duplicate samples from the flask at the end of the 24 hour period. Similar determinations were made on fresh medium as a basis for detecting net changes due to the activity of the organisms.

The cells produced several products from glucose. Significant quantities of both CO_2 and H_2 were evolved, but H_2 production was over twice that of the CO_2 production. Forty nine percent of the total acid extracted from the growth medium was lactic acid. Acetic and succinic acids were found in approximately equimolar concentrations, accounting together for 43 percent of the acid end products. Formic acid accounted for the remainder of the organic acid. On a molar basis, the production of either CO_2 or H_2 exceeded that of either acetate or succinate or a combination of the two. For each glucose equivalent of substrate utilized there was in excess of one CO_2 and 3 H_2 evolved.

The above analyses showed that 67 percent of the initial glucose in the medium was used by the cells. Determinations of the organic acids in the medium before and after trichomonad growth

Table 1. Fermentation balance of *Trit. augusta** in continuous culture.

I. Substrates and extracellular products.

Compounds	$\mu\text{M/hr.}$	$\mu\text{M C}$	Oxidation Value	Oxidized Product	Reduced Product
Glucose used	9.2	55.2	0		
Pyruvate used	13.0	<u>39.0</u>	+1	13	
Total used		<u>94.2</u>			
CO ₂	23.0	23.0	+2	46.0	
H ₂	55.0		-1		55.0
Formate	1.5	1.5	+1	1.5	
Acetate	3.8	7.6	0		
Lactate	9.8	26.7	0		
Succinate	4.0	<u>16.0</u>	+1	<u>4.0</u>	
Totals		<u>74.8</u>		<u>51.5</u>	<u>55.0</u>
				<u>-13.0^a</u>	
				38.5	

Carbon recovery = 79.9% O/R = 0.70

*) 1.2×10^6 cells were present per ml of growth medium. The flow rate of medium into the growth flask was 5.4 ml/hr.

a) The oxidized substrate was subtracted from the end products since it entered into the system already in an oxidized state.

II. Interacellular products.

Malate	0.227	0.910	+2	1.82	
Fumarate	0.340	1.360	+2	2.72	
Succinate	0.097	0.390	+1	.39	
α -Ketoglutarate	0.0	-	-	-	
iso-Citrate	0.0	-	-	-	
Citrate	0.0	-	-	-	
Oxaloacetate	0.0	-	-	-	
Lactate	0.146	0.437	0		
Acetate	0.470	0.940	0		
Formate	0.421	0.421	+1	0.421	
Pyruvate	0.400	1.200	+1	1.200	
Glycogen	0.300	<u>1.800</u>	0		
Subtotals		<u>7.458</u>		<u>6.551</u>	
Totals (part I + II)		82.26		45.05	

Carbon recovery = $82.26/94.20 = 87.3\%$ O/R = $45.05/55.0 = 0.$

showed a definite pyruvate utilization from the fresh medium. Thus pyruvate was considered as a substrate in addition to glucose. Computations from the continuous culture apparatus gave a carbon recovery of 79.9 percent and an O/R ratio of 0.70 (Table 1, part I). Table 1 (part II) shows the results of the carbon recovery when the intercellular organic acids were added as end products to the total balance. The intercellular products were: malate, fumarate, succinate, lactate, acetate, formate, pyruvate and glycogen. The limiting concentration of glucose used kept glycogen reserves low. Incorporating the intercellular organic acids into the overall balance increased the carbon recovery to 87.3 percent and the O/R ratio to 0.819.

B. Manometric System: A fermentation balance was determined using non-growing cells in Warburg manometer flasks. A population of 2.4×10^7 trichomonads (0.624 mg N/ml) was suspended in 3 ml of 80% KRP buffer (pH 6.6) containing 200 μ M of glucose. Control flasks lacking glucose were employed to correct for endogenous gas and acid production. Acetic, lactic and succinic acids were the only organic acid end products detected (Table 2). In this carbon balance acetate and lactate were produced in approximately equimolar concentrations and constituted 91 percent of the total acid. Succinate accounted for only 9 percent of the acid. On a molar basis CO_2 and H_2 production was high. The CO_2 exceeded the acetate produced and the H_2 production was higher than the total for all organic acids. Only 6.7 percent of the glucose used was stored as glycogen. The calculated carbon recovery accounted for 95.7 percent of the glucose used by the trichomonads.

Table 2. Glucose fermentation balance of Trit. augusta* in the Warburg apparatus.

Compound	mmH ₂ /mmH Glucose	mmH ₂ C	Oxidation Value	Oxidized Product	Reduced Product
Glucose used	1.00	300	0		
CO ₂	0.927	46.35	+2	92.7	
H ₂	2.370		-1		118.3
Acetate	0.783	78.30	0		
Lactate	0.750	112.50	0		
Succinate	0.150	30.00	+1	7.5	
Glycogen	0.067	<u>20.10</u>	0	<u> </u>	<u> </u>
Totals		287.25		100.2	118.3

Carbon recovery = 95.7%

O/R = 0.85

*) 2.4×10^7 cells per flask in phosphate buffer pH 6.6

The O/R value was 0.85 suggesting either the loss or anabolic incorporation of an oxidized end product.

11. Acetate, Hydrogen and Carbon Dioxide Production.

Both a clostridial-type of phosphoroclastic split and an Escherichia coli type split of pyruvate have been proposed to occur in trichomonads (23, 40). The clostridial-type split would form acetyl phosphate and CO_2 from pyruvate and H_2 would be evolved by the action of hydrogenase on reduced NAD without formate as an intermediate product. In the E. coli-type of split acetyl phosphate is also formed but formate acts as the intermediate in the production of both CO_2 and H_2 .

A. Acetyl Phosphate Production: If acetate is formed from acetyl phosphate a molecule of ATP could be formed for each acetate molecule. An attempt to demonstrate acetyl phosphate formation by cell extracts of Trit. augusta showed 14 times as much acetyl phosphate formed with pyruvate as a substrate as compared to endogenous, pyruvate free controls (Table 3). These results suggest that acetyl phosphate is an intermediate in the pathway for CO_2 and H_2 production in Trit. augusta.

B. The Hydrogenlyase System: This system has been shown to consist of two different enzymes (34) coupled together as an electron transfer system. Formic dehydrogenase catalyzes the formation of CO_2 and a reduced electron acceptor from formate. Hydrogenase forms H_2 from the reduced electron acceptor.

The manometric assay for formic dehydrogenase showed a slight lag in the release of CO_2 from the substrate, possibly due to CO_2 retention in the buffer (Figure 1). Gas evolution ceased after 20

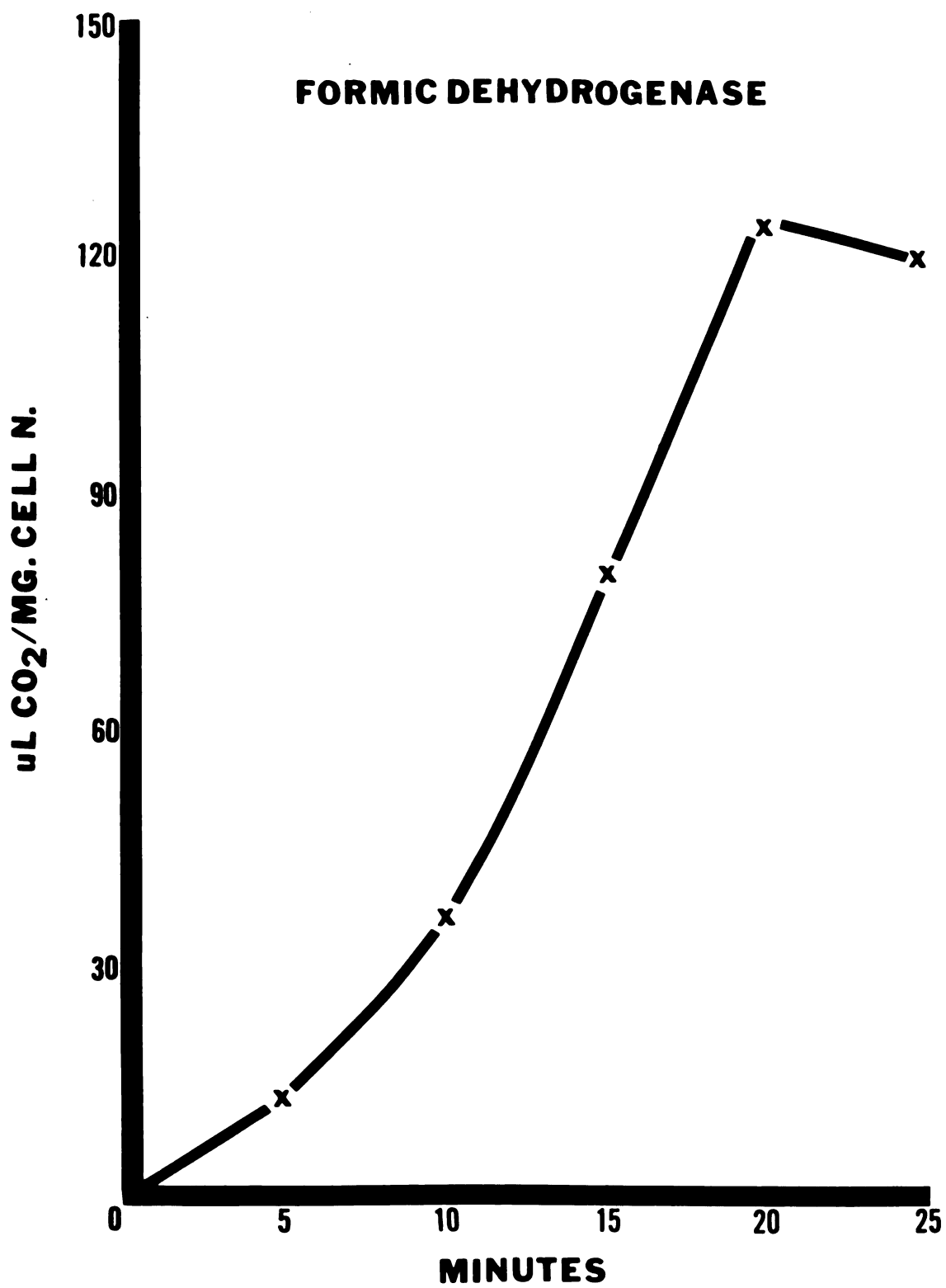
Table 3. Demonstration of acetyl phesphate production by Trit.
augusta.

Sample	O. D. at 540 mu	μM_5^{c} Acetyl $\text{PO}_4/\text{mg cell N}^*/\text{hr.}$
Control (no pyruvate)	0.003	0.284
Complete System ^a	0.052	<u>3.840</u>
Total		3.556

*) Homegenate added contained 0.648 mg cell N.

a) The complete system for acetyl phesphate analysis contained: 0.5 ml of 0.1M acetate buffer (pH 5.4), 0.5 ml of hydroxylamine solution (0.25 ml 4M hydroxylamine-HCl + 0.25 ml 3.5M NaOH), 0.5 ml cell extract and 0.5 ml 0.05M sodium pyruvate (sidearm). The endogenous system contained no pyruvate.

Figure 1. Formic dehydrogenase activity of a cell homogenate of Trit. augusta. Each Warburg flask contained: 1.6 ml. 0.065M phosphate buffer pH 6.8, 0.5 ml. cell homogenate, (0.636 mg cell N) 0.5 ml. 0.4M sodium formate (sidearm 1), 0.4 ml. (32uM) benzyl viologen (sidearm 2). Control: formate replaced with buffer. Gas phase; nitrogen. Carbon dioxide evolution measured for 25 minutes at 30°C.



minutes. A similar cessation of enzyme activity was noted by Lindblom after 30 minutes (23). A total of 120 ul of CO_2 were liberated from sodium formate in 20 minutes at 30 C. The formate free control showed no CO_2 evolution.

The next step after finding formic dehydrogenase activity was to determine if there was an active hydrogenase in these trichemenads. Hydrogen evolution was followed for 25 minutes with methyl viologen (reduced immediately before use by sodium hydrosulfite) acting as the source of electrons, i.e. substrate for the hydrogenase. Nearly 65 ul of H_2 were released from the reduced methyl viologen in the first 20 minutes of the reaction (Figure 2). Gas evolution ceased after 20 minutes as observed for CO_2 production from formate by formic dehydrogenase. In the control lacking reduced methyl viologen no H_2 was released from the system, which showed that the cysteine present in the system was not the source of H_2 for hydrogenase.

The third step in this series of experiments was to demonstrate the activity of the complete formic hydrogenlyase system. An artificial electron acceptor was not necessary in the complete reaction when non-dialyzed homogenate was used in the assay. In Figure 3 one can see that the complete system did indeed function. There was 175 ul of H_2 released from pyruvate for each mg of cellular nitrogen. Once again a drop in the gas evolution was observed after 20 minutes. There did not appear to be any lag in the formation of H_2 gas by the cell extract as reported by Lindblom with homogenates of Trit. fetus, Trit. suis and P. gallinarum (23). The specific activities of the above mentioned enzymes were

Figure 2. Hydrogenase activity of an extract of Trit. augusta cells. Each Warburg flask contained: 1.0 ml. 0.0625M phosphate buffer pH 6.45, 0.5 ml. cell extract (0.636 mg cell N), 0.4ml. of 32uM methyl viologen and 0.4 ml. of 80uM sodium hydrosulphite added from the sidearm just prior to start of experiment. The centerwell contained 0.2 ml. of 20% KOH. The control lacking methyl viologen showed no H₂ evolution. The Gas Phase was nitrogen. Hydrogen evolution was measured at 5 minute intervals for 25 minutes.

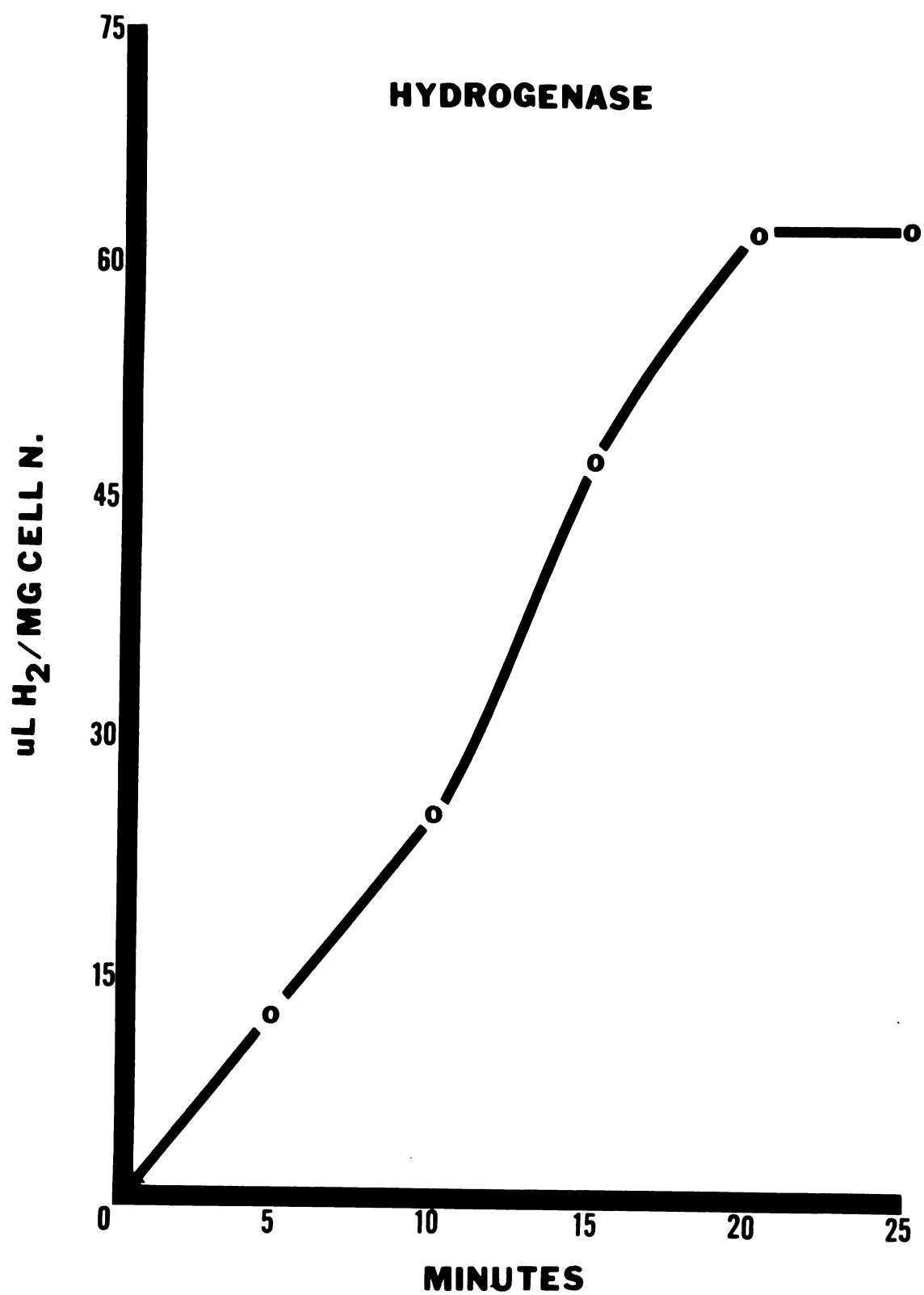
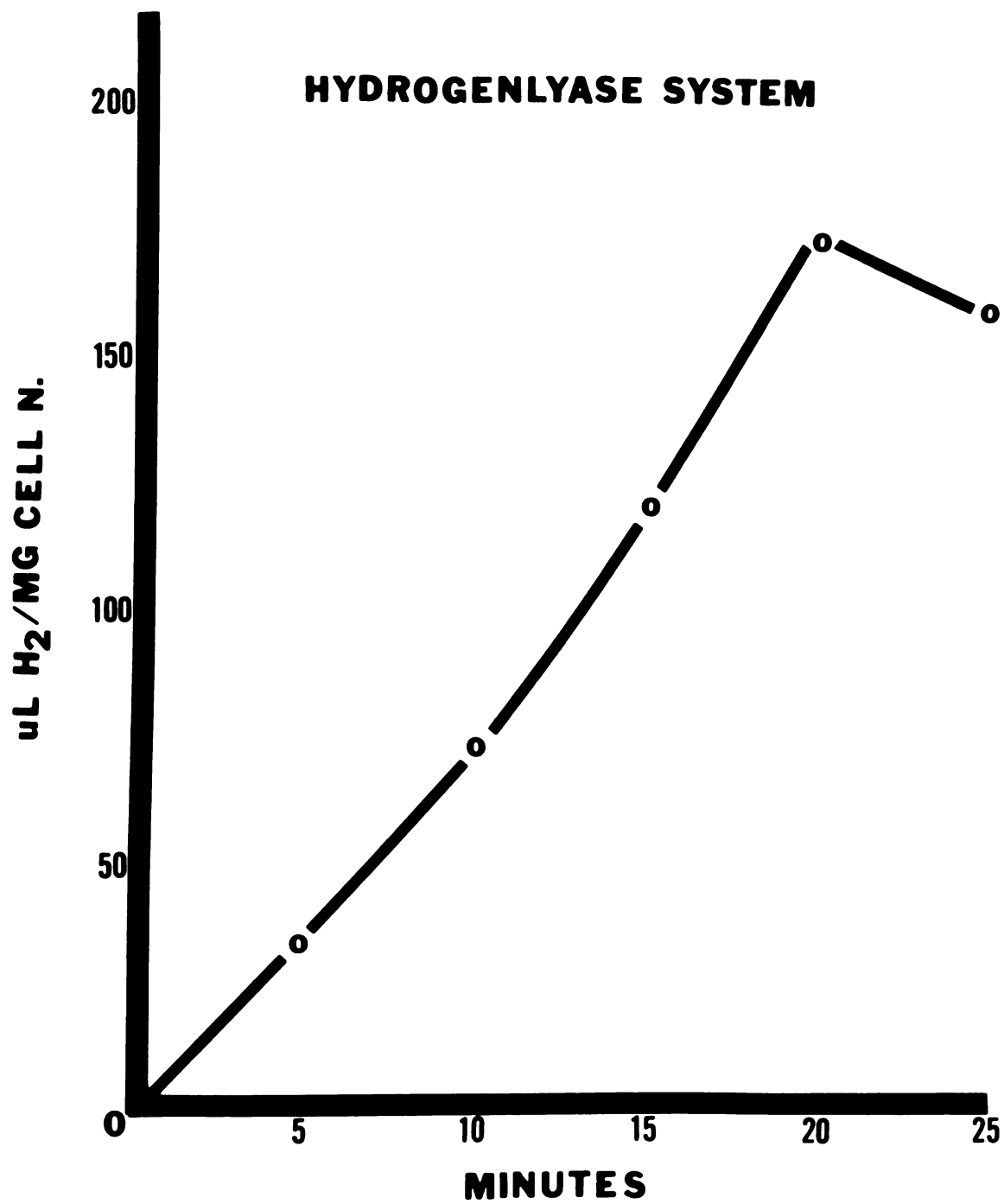


Figure 3. Formic hydrogenlyase activity in cell extracts of Trit. augusta. Warburg flasks contained: 1.8 ml. 0.065M phosphate buffer at pH 6.45, 0.5 ml. cell extract (0.636 mg. cell N), 0.5 ml. of 0.4M sodium pyruvate in the sidearm and 0.2 ml. of 20% KOH in the centerwell. Prior to the addition of pyruvate no gas was liberated over a 15 minute period. This served as the control. The gas phase was N₂.



listed in table 6.

The above detection of a complete formic hydrogenlyase system in cell extracts of Trit. augusta coupled with the determination of acetyl phosphate (Table 3) and formate (Table 1, part I and II) favors the presence of an E. coli-type of pyruvate split in this protozoan.

III. Demonstration of a Pathway for Succinate Formation.

Succinic acid is a known end product of glucose fermentation for most of the trichomonads studied, but previous investigations have failed to show the pathway for its formation. It seemed that a determination of organic acids in the cells of Trit. augusta might give some indication of the enzymes present in the pathway leading to the production of succinate. A separation of ether soluble material from cell extracts by celite chromatography revealed peaks for malate, fumarate, succinate, acetate, formate, lactate and pyruvate (table 1, part II). Only small amounts of succinate, lactate and malate were recovered with acetate, formate and pyruvate making up the bulk of the acids. The presence of acetate and formate was accounted for in the phosphorelastic split of pyruvate. Lactate probably was formed by the reduction of pyruvate by lactic dehydrogenase. Since succinate is the end product of the pathway and malate and fumarate were found in the cells all the intermediates were present for a reversal of part of the Kreb's cycle. Previous investigations had demonstrated malic enzyme (59) and succinic dehydrogenase (43) in trichomonads.

The quantity of pyruvate in the cells and the demonstration of malic enzyme suggested that pyruvate acted as the acceptor for

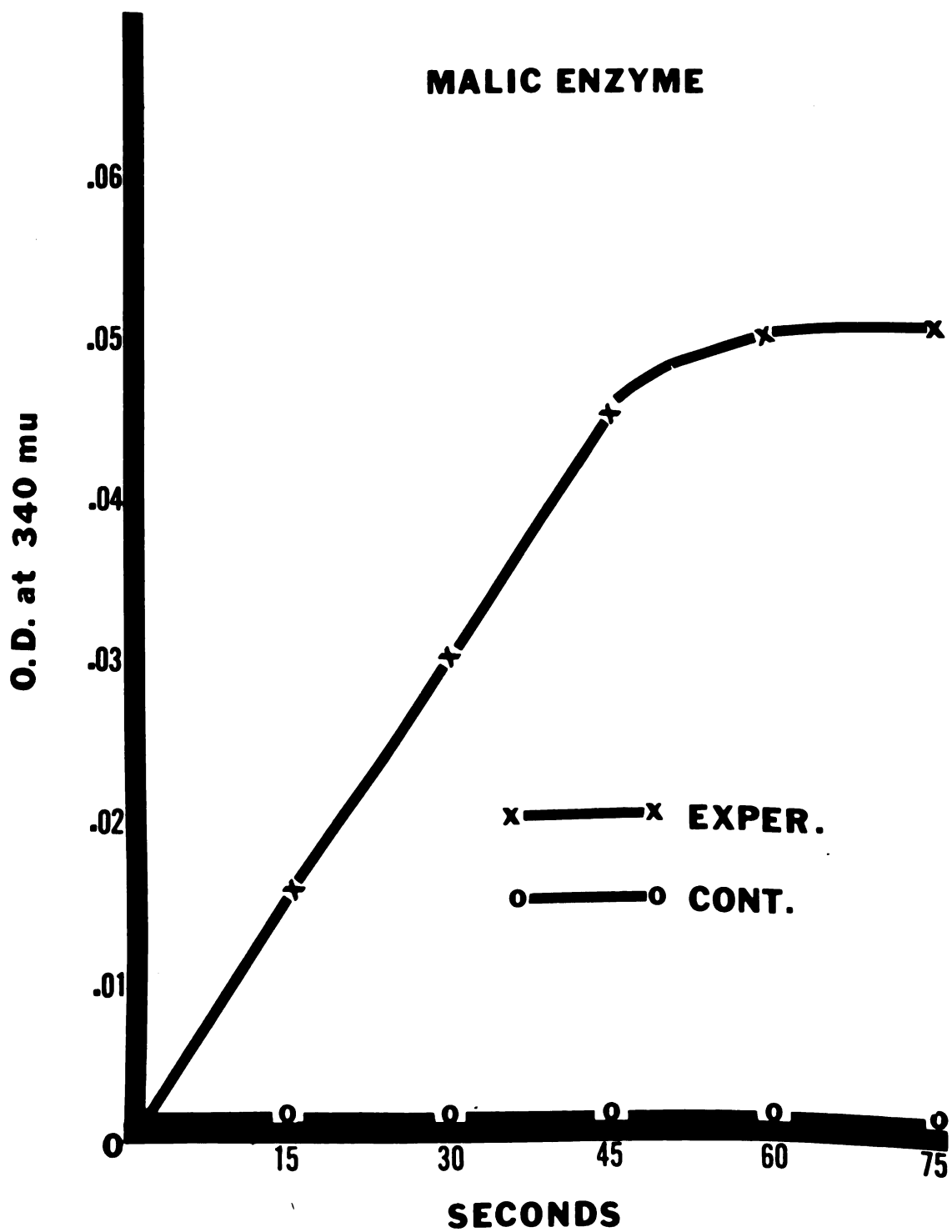
CO_2 in the formation of malate. Both malic enzyme and pyruvate carboxylase are capable of fixing CO_2 to pyruvate. A third enzyme, phosphoenolpyruvate carboxylase, fixes CO_2 to phosphoenolpyruvate. Neither pyruvate carboxylase or phosphoenolpyruvate carboxylase both of which catalyze the formation of oxaloacetate, were detected in cell extracts of Trit. augusta. The fact that oxaloacetate was not detected in cell homogenates of Trit. augusta (Table 1, part II) supports the probable importance of malic enzyme in CO_2 fixation.

The assay for malic enzyme by the method described by Ochoa proved successful (31). The increase of optical density (O.D.) at a wave length of 340 mu showed the formation of reduced NADP (Figure 4) indicative of malic enzyme activity. Controls lacking malate, NADP or cell homogenate were negative.

In order to form succinate by a reversal of the tricarboxylic acid cycle, malic acid must be converted to fumarate by fumarase through the removal of one water molecule. Lindblom was unable to demonstrate fumarase in Trit. foetus, Trit. suis and P. gallinarum (23). Attempts to detect fumarase in cell homogenates of Trit. augusta failed when the method described by Massey (27) was used with either fumarate or malate as substrates. Another assay was devised which utilized the coupling of two reactions that normally occur in the TCA cycle. The complete procedure is outlined in the materials and methods and the reactants listed in figure 5. In brief, this entailed the conversion of fumarate to malate and then the oxidation of malate to oxaloacetate by malic dehydrogenase which was added to the reaction mixture. Phenazine methosulphate,

Figure 4. Malic enzyme activity in an extract of Trit. augusta. The cuvette contained: 0.3 ml. 0.025M glycylglycine buffer pH 7.4, 0.06 ml. 0.05M MnCl₂, 0.2 ml. 0.000675M NADP, 0.05 ml. 0.03M L-malate pH 7.4, 1 ml. dialyzed cell homogenate, and water to adjust final volume to 3 ml. The reaction was followed spectrophotometrically by determining the optical density at 340 mμ every 15 seconds. Three controls (one lacking malate, one lacking NADP and a third without cell homogenate) showed no increase in optical density.

MALIC ENZYME



substituted for NAD, acts as a H^+ acceptor in the conversion of malate to oxaloacetate. The two electrons involved were passed to a tetrazolium salt with a resulting change in its O.D. at 650 m μ (Figure 5). In the control which lacked sodium fumarate in the reaction mixture, there was no change in absorption. Unfortunately, the spectrophotometer was not adjusted to give a zero reading for the inactive reaction mixture.

The final step in demonstrating the pathway leading to the formation of succinic acid was the reduction of fumarate by succinic dehydrogenase. The assay for this enzyme system was carried out spectrophotometrically at 400 m μ by the method described by Bonner (5). The procedure and results are outlined with Figure 6. The fumarate free control showed no measurable activity.

The obligate anaerobe, Micrococcus lactilyticus, contains an enzyme, fumarate reductase, which catalyzes the reduction of fumarate to succinate far faster than the reverse reaction (35). It is relatively insensitive to malonate inhibition (57). An attempt was made to determine if the enzyme in Trit. augusta was similar to that noted for M. lactilyticus by testing the inhibition of the reaction by malonate. There was a definite decrease in succinate production from glucose in the presence of malonate (Figure 7). Only a trace of succinate could be measured in the sample eluted from a celite chromatography column. It was also obvious there was an accumulation of fumarate in the trichomonad cells treated with malonate (Figure 7) and that the succinic dehydrogenase in Trit. augusta was susceptible to malonate at the concentration employed. The specific activities of the enzymes

Figure 5. Activity of fumarase in cell homogenates of Trit. augusta. Reaction mixture: 0.5 ml. 0.017M sodium fumarate, 0.5 ml. 0.033M phosphate buffer pH 6.8, 0.1 ml. 0.0015M NAD, 3 ml. 1% solution of Triphenyltetrazolium chloride, 0.5 ml. phenazine methosulphate (1mg./10 ml.), and 1.0 ml. dialyzed cell homogenate. A three ml. aliquot was placed into a cuvette and 20 units of malic dehydrogenase was added to it. The reaction was then measured at one minute intervals at a wavelength of 650 mu. The control system was composed of the above reaction mixture omitting the sodium fumarate.

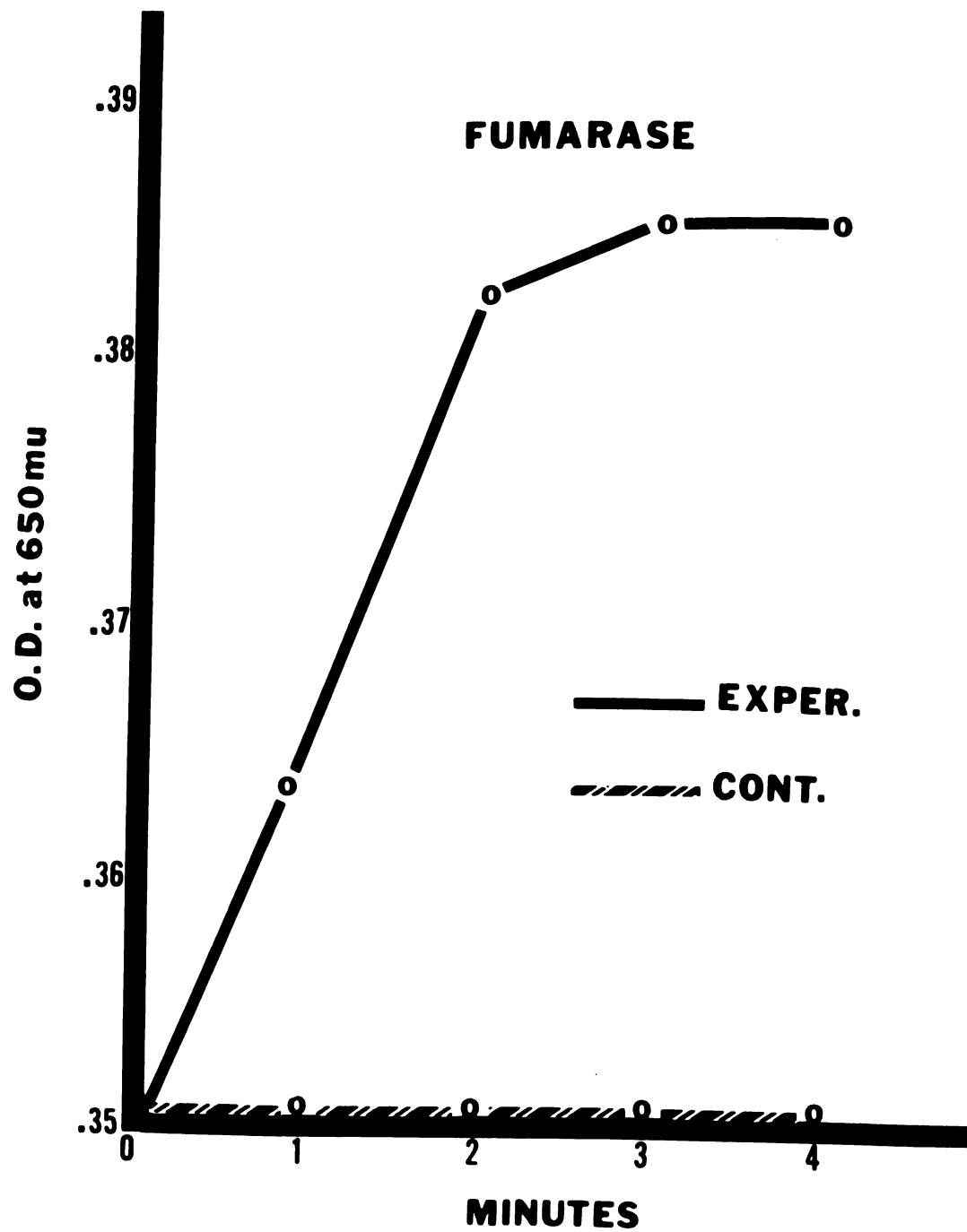


Figure 6. Succinic dehydrogenase in a crude extract of Trit. augusta. The cuvette contained: 0.3 ml. 0.1 M KCN, 0.3 ml. 0.01M $K_3Fe(CN)_6$, 0.2 ml. 0.2M sodium succinate 2 ml. 0.15M phosphate buffer pH 7.2. The reaction was initiated by the addition of 0.2 ml. of dialyzed cell homogenate. The change in optical density was followed spectrophotometrically at 400 m μ at 15 second intervals. The control system contained the all above components except sodium succinate.

SUCCINIC DEHYDROGENASE

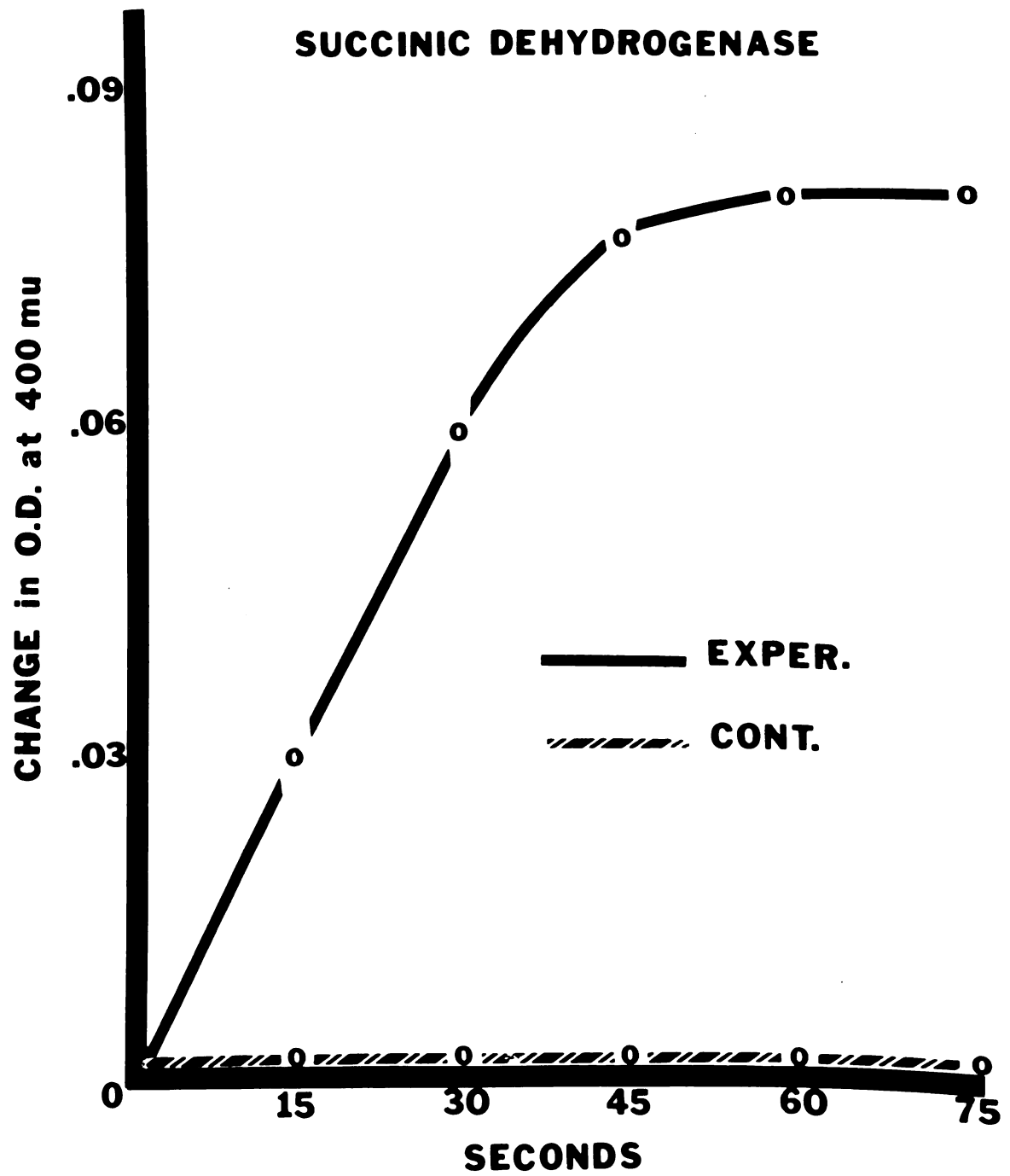
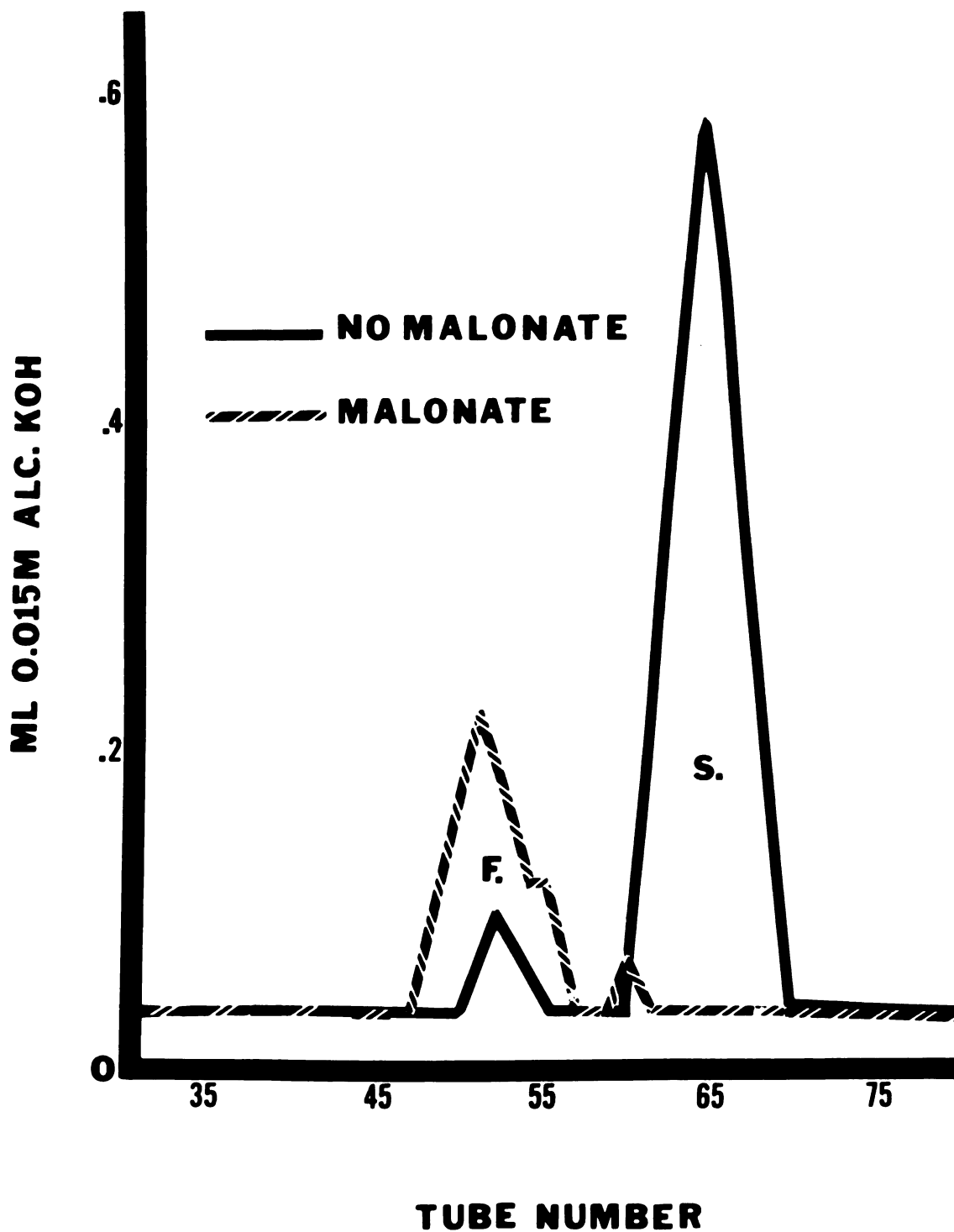


Figure 7. Malonate inhibition of succinate formation in whole cells of Trit. augusta. Each Thunburg tube contained: 1 ml 0.02M glucose, 1 ml of a cell suspension containing 3×10^6 cells per ml, 0.1 ml malonate and 7.9 ml 0.1M phosphate buffer pH 6.8. The control consisted of the above mixture minus malonate. The Thunburg tubes were alternately placed under vacuum and flushed with oxygen-free nitrogen three times. They were then incubated one hour at 30 C. The cells were then removed from the reaction mixture by centrifugation and the supernatant extracted with ether and separated on a celite column. The concentration of fumarate and succinate are given with and without malonate in the reaction system.

Key: F = fumarate, S = succinate.

INHIBITION OF SUCCINATE FORMATION BY MALONATE



involved in the succinate pathway are given in table 6.

IV. Oxidation-Reduction Systems Associated with Lactic and Succinic Acid Production and Adenosine Triphosphate Formation.

Lactic dehydrogenase has been reported in T. vaginalis (2, 60). Using the method of Kornberg (19) an attempt was made to demonstrate lactic dehydrogenase in Trit. augusta by following the oxidation of NADH+H to NAD with sodium pyruvate as substrate. The results showed the enzyme to be very active in this organism (Figure 8). When either pyruvate or NADH was omitted from the reaction mixture no enzyme activity occurred.

Flavin adenine dinucleotide is usually the flavin associated with succinic dehydrogenase. To determine if this coenzyme was present, homogenates of 1×10^7 Trit. augusta organisms and standard solutions of FAD and riboflavin were chromatographed on paper. Spots were located by their fluorescence and the calculated Rf values of the unknowns were found to be identical to those obtained for standard solutions of FAD and riboflavin (Table 4).

It next remained to determine if FAD participated in the reactions associated with the formation of succinate. An assay was devised whereby reduced NAD and oxidized FAD were added to a cell extract of Trit. augusta to detect any interaction of these two coenzymes. The complete assay contained added FAD, ADP, NADH, phosphate buffer (pH 6.8) and cell homogenate. The oxidation of NADH was followed spectrophotometrically at 340 mμ at 15 second intervals for one minute (Figure 9). The oxidation of NADH appears to be dependent on the presence of ADP and inorganic phosphate. For the

Figure 8. Lactic dehydrogenase activity of an extract of Trit. augusta. The cuvette contained: 0.1 ml 0.01M sodium pyruvate, 0.1 ml 0.002M NADH, 1.0 ml of 0.1M phosphate buffer pH 7.4 and 0.2 ml dialyzed cell extract. Two controls were prepared: one lacking pyruvate and a second lacking NADH. The oxidation of the reduced NAD was followed spectrophotometrically at 340 mμ with the change in O.D. recorded every 15 seconds. Neither control showed a detectable change in O.D.

LACTIC DEHYDROGENASE

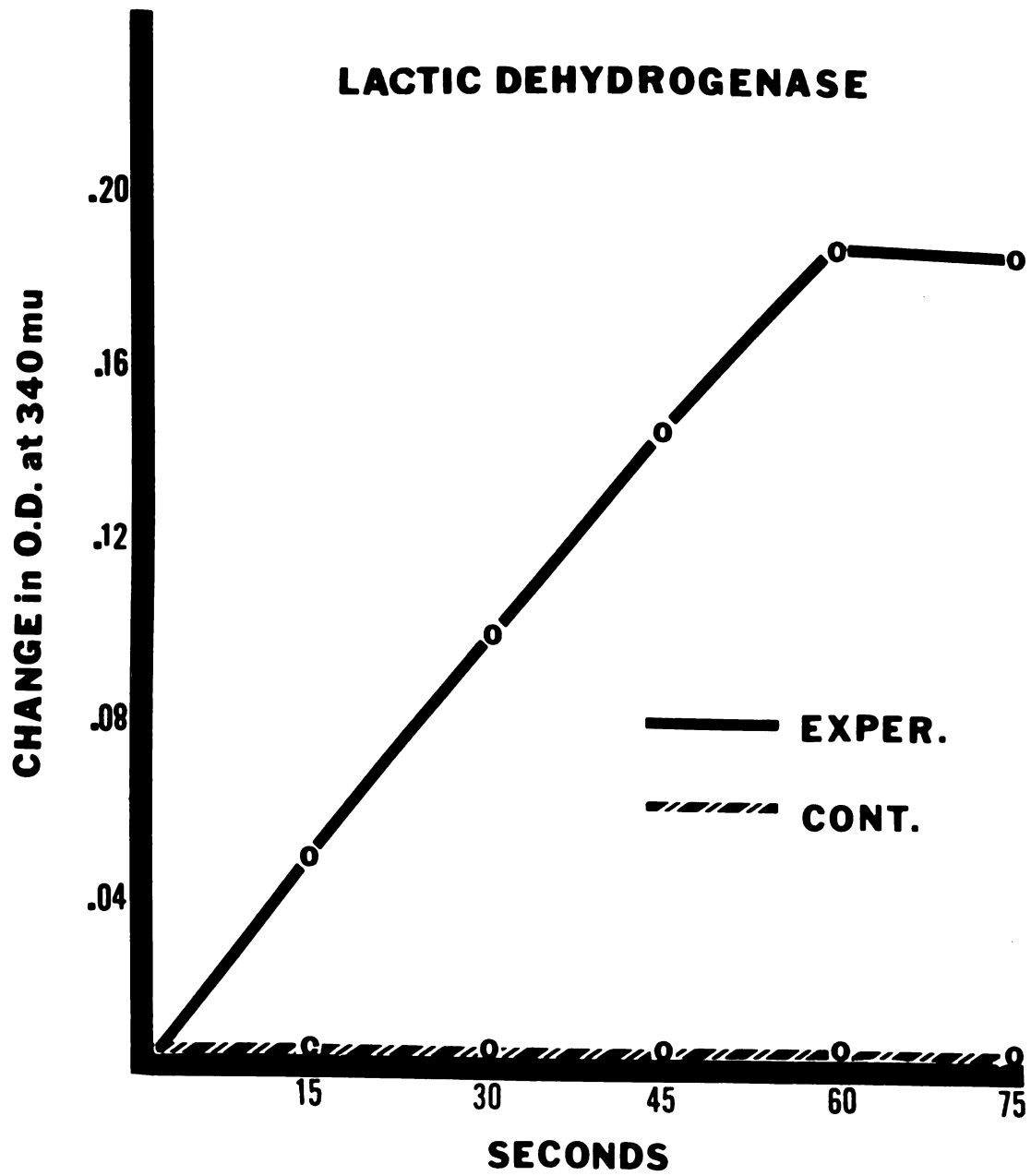


Table 4. Evidence for the presence of riboflavin, FAD and ATP in Trit. augusta as determined by paper chromatography.

Controls	Rf Values*	Cell Homogenate	Rf Values*
10 lambda 0.002M Rb	0.81	80 lambda	0.81
10 lambda 0.002M FAD	0.15	80 lambda	0.15
10 lambda 0.002M ATP	0.48	80 lambda	0.49

*) Rf values were determined by observing the fluorescence of the flavins and the visual UV. quenching of ATP using a Mineralite lamp.

Table 5. Amount of ATP resulting from electron transport associated with succinate production.

Reaction Mixture	As*257mu	Change in As 340mu/min/ml.	uM ATP*/ml.	uM NADH ¹ Oxid./ml.	$\frac{\text{uM ATP}}{\text{uM NADH Oxidized}}$
Exogenous ^a	0.105	0.042	0.071	0.071	
Endogenous ^b	0.015	-----	0.010	-----	
Exo. - Endo.	0.090	0.042	0.061	0.071	$\frac{0.061}{0.071} = 0.847$

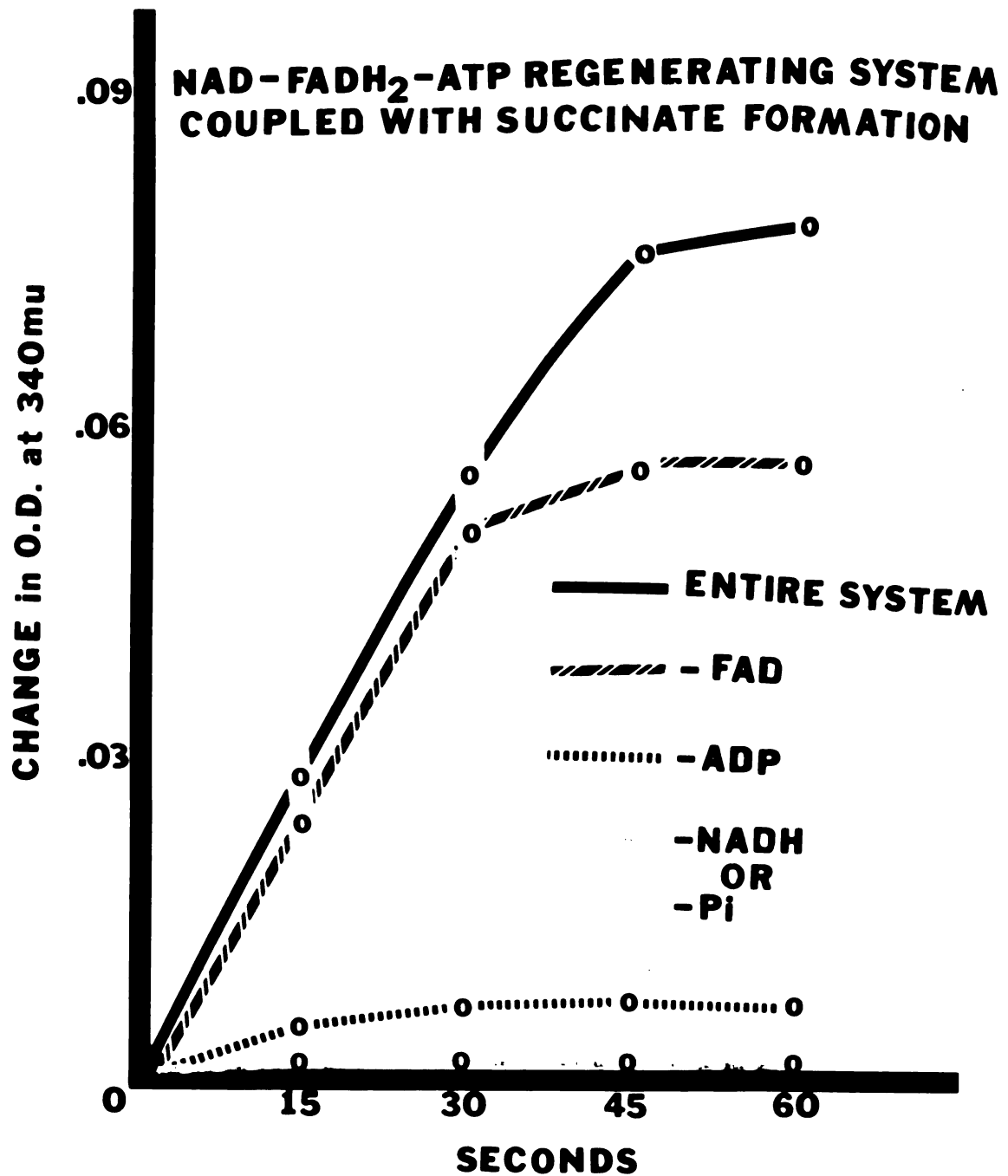
a) Exogenous Reaction Mixture: 1.7 ml. 0.1M phosphate buffer pH 6.8, 0.2 ml. of 0.002M ADP, 0.002M FAD, and 0.002M NADH and 0.7 ml. dialyzed cell extract.

b) Endogenous Mixture: Above minus the reduced NAD.

*) ATP was separated from the reaction mixtures by paper chromatography as described in the methods section. Millimoles of ATP were determined using the following equation: $A_m = A_s/C$; where A_m = molar absorptivity index; A_s = absorbancy and C = concentration in mM/liter.

¹) The amount of NADH oxidized per min. per ml. was determined from the change in absorbancy at 340 mu using the same equation given above.

Figure 9. Activity of enzyme(s) involved in the terminal respiration of Trit. augusta. In cuvette: 0.1 ml. 0.002M NADH, 0.1 ml. 0.002M FAD, 0.1 ml 0.002M ADP, 2.2 ml 0.1M phosphate buffer pH 6.8 and 0.5 ml. cell homogenate dialyzed in TES buffer. Controls: 1) no NADH, 2) no FAD, 3) no ADP and 4) no inorganic phosphate. The oxidation of NADH was determined spectrophotometrically at 340 mμ at 15 second intervals for one minute.



first 30 seconds the addition of oxidized FAD had no effect on the rate of NADH oxidation, but after 30 seconds FAD is required for optimum activity. Because the FAD-NADH oxidation reduction system became inoperative in the absence of ADP and inorganic phosphate it appeared as though a reaction similar to the first step of an oxidative phosphorylation scheme might be present in this anaerobic protozoan.

To determine if indeed there was a production of ATP in the above reaction paper chromatographs of the complete reaction mixture were compared to mixtures containing no NADH and to ATP standards. The Rf values of the spots from both reaction mixtures were identical to the Rf values of the standard ATP (Table 4). A 7-fold greater yield of ATP was found in the complete mixture than in the endogenous system (Table 5). A quantitative comparison showed that for every NADH molecule oxidized 0.847 molecules of ATP were formed. The specific activity of the enzymes involved is listed in table 6.

In comparing the specific activities of all the enzymes studied in this investigation lactic dehydrogenase is by far the most active. Succinic dehydrogenase, the second in activity is only half as active as lactic dehydrogenase. The activities of the other enzymes were much lower than succinic dehydrogenase.

Table 6. Specific activity of enzymes associated with the production of end products by Trit. augusta.

<u>Enzyme</u>	<u>Specific Activity</u>
<u>Hydrogen Production</u>	
Formic Dehydrogenase	3.5*
Hydrogenase	2.5
Formic Hydrogenlyase	7.0
<u>Succinic Acid Production</u>	
Malic Enzyme	6.98 ^a
Fumarase	2.0
Succinic Dehydrogenase	69.8
<u>Systems for Oxidation of NADH</u>	
Lactic Dehydrogenase	107.5 ^a
Terminal Respiration-Type System (redn. of FAD, oxid. of NADH and formation of ATP)	17.1
*) Specific activity based on the conversion of 1 μ M of substrate/ min./mg. cell N.	
a) Specific activity expressed as units/mg. cell N, where one unit of enzyme is defined as that amount required to cause a change in O.D. of 0.01/min.	

DISCUSSION AND CONCLUSIONS

This investigation showed that Trit. augusta was capable of metabolizing glucose to a mixture of end products which included acetate, lactate, formate, succinate, CO_2 and H_2 . Similar end products have been reported for other species of trichomonads (12, 18, 23, 37, 49, 58, 60). However, all the metabolic pathways involved in the formation of these end products are incompletely known except for that of lactic acid formation. Demonstrations of lactic dehydrogenase in T. vaginalis (2, 60) and in Trit. augusta in this study provide conclusive evidence that lactic acid can be formed by the reduction of pyruvate. This mechanism illustrates one means by which Trit. augusta can oxidize NADH so that this co-factor can recycle through the glycolytic pathway.

Gas and Acetate Formation.

Suzuoki and Suzuoki were the first investigators to detect formic dehydrogenase in a trichomonad (50). However, they were unable to show a complete hydrogenlyase system. Two different pathways for the formation of acetate, CO_2 and H_2 have been postulated, both similar to those found in certain facultative and obligate anaerobic bacteria (23, 40). Because Ryley could not obtain H_2 evolution from formate in homogenates of Trit. fetus under anaerobic conditions, he proposed that Trit. fetus must produce H_2 by a pathway similar to the phosphoroclastic-type split of pyruvate found in the Clostridia (40). Lindblom detected strong formic dehydrogenase activity, but only weak

hydrogenase activity in members of the Trit. fetus complex and P. gallinarum (23). He reasoned that the trichomonads produced H_2 like the Enterobacteriaceae because of their strong formic dehydrogenase activity and his inability to demonstrate the presence of a clostridial-type of reaction.

In this investigation both acetyl phosphate and formate plus the two enzymes of the formic hydrogenlyase system were detected in Trit. augusta. The theory of an Enterobacteriaceae - like phosphorelastic system being involved in trichomonad gas evolution seems to apply to Trit. augusta and probably to other trichomonads as well. The acetyl phosphate is most likely the precursor of the acetate produced as an end product by this protozoan. Bacteria such as the Enterobacteriaceae can hydrolyze acetyl phosphate to form acetate and high energy phosphate which reacts with ADP to form ATP. The presence of acetyl phosphate is presumptive evidence that Trit. augusta and other trichomonads may form ATP by this method. However, it is possible that other species of trichomonads have different or added mechanisms for $H_2 + CO_2$ evolution. An NADH oxidase system has been demonstrated in I. vaginalis which may serve to oxidize NADH with the release of molecular H_2 (26, 58).

Succinate Production.

None of the previous investigations on trichomonads have shown a complete pathway for succinic acid production. When Kunitake et al. used labeled glucose as a substrate for the growth of I. vaginalis they found a significant amount of the labeled carbon appearing in amino acids (21). The labeled carbon in a

succinate substrate appeared in CO_2 . They considered the labeling pattern as evidence for a Krebs's cycle even though they were unable to demonstrate the enzymes of the pathway in cell free systems. Two possible pathways have been suggested for the production of succinic acid (23). One possibility was the condensation of two acetate molecules (as proposed by Seaman (45) for *Tetrahymena*) derived from either a phosphoroclastic reaction or from the hexose monophosphate pathway. The second was the formation of malic acid from the fixation of CO_2 to lactate or pyruvate. Lindblom did not speculate on how succinate would be formed from malate other than to state that fumarate, though undetected, might exist in these protozoans (23).

After an earlier failure (58), Wellerson and Kupferberg found a mammalian type of malic enzyme in *I. vaginalis* (60). Ryley provided indirect evidence for CO_2 fixation in *Trit. fetus* by showing succinate formation was dependent on the presence of CO_2 (40). A 'malic type enzyme' was also detected in *P. gallinarum* which removed CO_2 from malate and left lactic acid as the end product (23). The inability of investigators to demonstrate fumarase in cell extracts of several trichomonads left the pathway for succinate production open to conjecture (4, 23). Seaman reported that cell extracts of *I. vaginalis* possessed a succinic dehydrogenase (43). This enzyme was not detected in whole cells of *I. vaginalis*, but Read admitted that the inability of succinate to permeate into intact cells could explain his negative results (37, 62).

A. Malic enzyme: The results of this investigation on *Trit.*

augusta supports previous observations that the malic enzyme is essential for malate formation. The enzyme found in this organism resembles the enzyme described for T. vaginalis in that it required reduced NADPH (60). The NADPH required by this system in vivo might come from the conversion of glucose-6-phosphate to 6-phosphogluconate by glucose-6-phosphate dehydrogenase, an enzyme which has been reported in the Trit. fetus complex and P. gallinarum (23). It is also possible that a mechanism for the interconversion of NADP by reduced NAD to NADPH may be present in these cells although no attempts have been made to demonstrate such a system. The malic enzyme demonstrated in Trit. augusta fixes CO_2 to pyruvate and not lactate. It is possible that the lactate detected by Lindblom, as the result of malate decarboxylation (23), actually resulted from the rapid reduction of pyruvate by a very active lactic dehydrogenase. His undialyzed preparations may have contained a sufficient concentration of NADH to facilitate lactate formation.

B. Fumarase: For succinate formation to occur by reversal of the Kreb's cycle the conversion of malate to fumarate by fumarase is essential. The demonstration of fumarase activity in Trit. augusta proved to be not only the most difficult but also the most essential contribution to the determination of the pathway for succinate formation. A coupled reaction was necessary to demonstrate fumarase activity because of the apparent need for strong electron acceptor such as phenazine methosulphate to make the reaction proceed to completion. The in vivo association of the enzyme with the succinic dehydrogenase system and its flavin

acceptor may serve to control its activity.

C. Succinic dehydrogenase: The demonstration of succinic dehydrogenase in Trit. augusta supported the report of this enzyme in I. vaginalis (43) and completed the list of enzymes essential for succinate formation by Trit. augusta. The strong inhibition of succinic dehydrogenase in this organism by malonate shows that the trichomonad enzyme resembles the mammalian enzyme more than the fumarate reductase reported for M. lactilyticus (35, 57). Malonate inhibition of succinate dehydrogenase will not stop the growth of trichomonads (46). Since malonate will inhibit trichomonad motility (40), perhaps the additional energy supplied by the terminal respiration system is in part responsible for active locomotion.

Electron Transport System Associated With Succinate Pathway.

Flavins have been detected in I. vaginalis (58) and indirect evidence has been reported for the presence of a flavoprotein terminal oxidase in this organism (4, 19, 37). An NADH oxidase has also been described for I. vaginalis that operates in the presence of oxygen (58). The significance of an NADH oxidase system that incorporates oxygen is difficult to evaluate since these trichomonads fail to grow in an aerobic environment.

Studies on Trit. augusta suggest that the oxidation of FADH_2 and the reduction of NAD occur jointly with the operation of the succinic dehydrogenase system. The coupling of these electron transport systems result in the anaerobic reduction of fumarate by NADH. Phosphorylation appears to be concurrent with succinate formation or the accompanying electron transfer. In this way the formation of succinate not only provides a means of reoxidizing

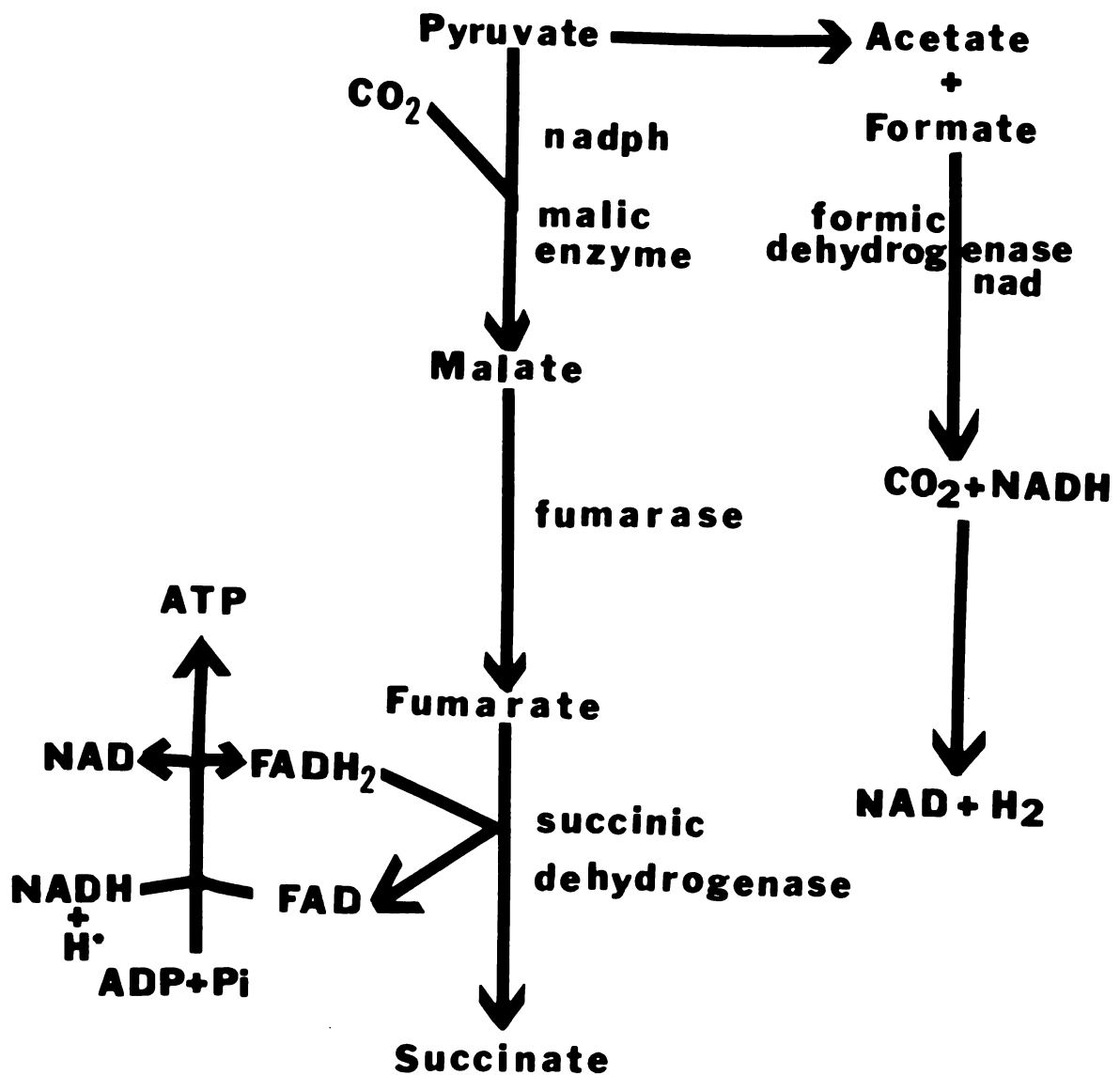
NADH, but also generates energy in the form of ATP with nearly one ATP produced for every NADH oxidized. It is possible that the NADH oxidase system proposed by Wellerson et al. for T. vaginalis (58) might require flavins for activity and be part of the electron transport system observed in Trit. augusta. Unfortunately, Wellerson and his co-workers did not look for ATP production in association with the oxidation of NADH. The proposed pathways for the formation of all end products except lactate from pyruvate and the accompanying electron transport systems are illustrated in figure 10.

Systems for succinate production similar to the one described for Trit. augusta have been observed in other parasites such as Trypanosoma cruzi and Ascaris lumbricoides. When grown anaerobically T. cruzi produces acetate, lactate and succinate from glucose by an identical pathway to that seen in Trit. augusta (39). Succinate is also produced by this organism under aerobic conditions (6). T. cruzi differs from Trit. augusta in that a complete Kreb's cycle has been shown to be functional as well as CO₂ fixation to form succinate. This organism has also been shown to possess a terminal respiration system with cytochromes (39).

Muscle strips of A. lumbricoides were observed to fix CO₂ to pyruvate to yield malate by means of the malic enzyme system. This system differs from that in Trit. augusta in that it can utilize either oxidized NAD or NADP as a co-factor, but shows higher activity with NAD (41). Both fumarase and succinic dehydrogenase are demonstrable in the worm (41). Fumarate was reported to be reduced by NADH to form succinate in conjunction with several flavo-

Figure 10. Proposed scheme for succinate production, hydrogen and carbon dioxide evolution and terminal respiration in Trit. augusta.

**PROPOSED SCHEME FOR SUCCINATE
PRODUCTION - HYDROGEN EVOLUTION
AND TERMINAL RESPIRATION IN TRI-
TRICHOMONAS AUGUSTA**



protein carriers. These carriers were reported to be succinic dehydrogenase and NADH dehydrogenase (18). Kmetec speculated that the coupling of these systems in the nematode would provide not only a means for oxidizing NADH, but also would generate ATP (18). It is this type of system which seems to be functioning in Trit. augusta. However, A. lumbricoides decarboxylates succinate to form propionate rather than excreting it as an end product (41).

The bacterium, Propionibacter arabinosum, also has the ability to form succinate and propionate by a similar pathway (64). P. arabinosum uses the enzyme phosphoenolpyruvate carboxylase to fix CO_2 to phosphoenolpyruvate to form oxaloacetate. Oxaloacetate is then converted to succinate by a reversal of the Kreb's cycle. However, preliminary work has shown that this pathway is not the major source of energy in this organism. In fact the fixation of CO_2 by P. arabinosum requires energy (64).

Fermentation Balances.

The end products produced by an organism gives an insight to the metabolic pathways that may be present and the probable electron acceptors and donors involved. It is for this reason the stoichiometry of the glucose metabolism of Trit. augusta was studied. There were some differences between the fermentation balances obtained from the continuous culture system and the Warburg apparatus, even though cells for both systems were grown in continuous culture. A trace of formate was detected in the extracts from the continuous culture medium but not in the Warburg sample. The formate probably came from cells damaged by the impeller of the continuous culture apparatus.

A large difference was observed between the quantity of succinate formed in continuous culture and that measured in the Warburg balance. Manometric experiments using Trit. foetus without CO_2 in the gas phase showed low yields of succinate (40) similar to the results obtained with Trit. augusta. Since the trichomonads produce an excess of CO_2 , a low partial pressure of this gas would still exist in the fluid phase without adding CO_2 to the gas phase. In the continuous culture system the greater volume of fluid and a presumed reduced rate of exchange between the liquid and gas phase may have kept higher CO_2 tensions in the medium. This may have promoted succinate production in spite of the constantly changing N_2 atmosphere in the growth flask.

Lactate production was high in all fermentation balances. The reason Trit. augusta produces such large amounts of lactate, when the formation of succinate appears to be more profitable, is not known. Lactate production by means of the glycolytic pathway is however associated with rapid growing organisms. For example, homofermentative lactobacilli in a complex medium develop so rapidly that in 16 hours or less growth is limited by the amount of lactate accumulated (63). Possibly lactate is produced initially by Trit. augusta to promote rapid growth, and after there is a certain accumulation of lactate or other limiting conditions exist in the medium, metabolism is shifted to produce acetate and succinate to gain more energy (ATP) for cellular processes. Lindblom stated that, "the trichomonads he studied produced more acid during the early stages of their growth and since acid production points to an anaerobic metabolism, perhaps in

young cultures the glycolytic pathway is preferred" (23). Read and Rothman reported that T. vaginalis after being cultured for long periods of time tended to produce greater amounts of lactate (38). Perhaps the growth of trichomonads under artificial conditions is selective for organisms which grow most rapidly, i.e. those which produce the most lactic acid.

The quantities of CO_2 and H_2 reported in both carbon balances were too high on a molar basis for the amount of acetate formed if all CO_2 and H_2 is formed via the breakdown of pyruvate to acetate, CO_2 and H_2 . It is possible that these gases might arise from other pathways. A breakdown of amino acids present in the growth medium might be one such mechanism. There are large quantities of glutamic and aspartic acids present in the serum (17) and in the trypticase of Diamond's medium (Baltimore Biological Lab. assay, unpublished data). Perhaps these amino acids are decarboxylated by Trit. augusta and in the process CO_2 released. Trit. fetus has been shown capable of adsorbing amino acids from medium and maintaining them as a pool of free acids (17). Some of the CO_2 and H_2 produced in the Warburg balance may come from this amino acid pool. Peck Jr. and Gest have speculated from their data on hydrogenase that more than one type may exist, or that perhaps the enzyme is double-headed with two prosthetic groups (33). They proposed that one prosthetic group might mediate electron transport to physiological acceptors of relatively high redox potential, while the other might be concerned with reduction of electron acceptors of low potential and/or with the formation of molecular H_2 . The hydrogenase in Trit. augusta may

not be specific for the removal of H_2 from formate. It might also form H_2 from amino acids such as the cysteine added to the medium to aid anaerobiosis and from reduced co-factors such as NADH, $FADH_2$ and NADPH. The NADH or a flavin oxidase reported in I. vaginalis may be identical to the hydrogenase of Trit. augusta (3, 58, 62). There is also the possibility that other unidentified pathways exist for CO_2 and H_2 evolution which are responsible for the high gas production.

Ryley followed the anaerobic fermentation of intercellular glycogen by Trit. fetus manometrically (40). He obtained a fermentation balance which contained acetate, lactate, succinate, H_2 and negative CO_2 . His carbon recovery was 83% and the O/R ratio was 1.0. The amount of succinate produced was three times greater than the acetate formed in a gas phase of 95% N_2 and 5% CO_2 . It appears that Trit. fetus can use stored glycogen to form succinate and re-cycle NAD, and that it is not dependent on lactate production. Since the balances on Trit. augusta were done using an external glucose substrate it is difficult to compare these results with those of Ryley on Trit. fetus.

Conclusions:

The present work illustrates some of the pit falls in studying the fermentation characteristics of an organism using a complex undefined medium. However, the presence of lactic dehydrogenase, acetyl phosphate, the formic hydrogenlyase system and the intermediates and enzymes necessary for succinate do indicate the pathways in which the major end products of Trit. augusta are formed.

The reduction of fumarate and the concurrent oxidation of NADH appear to serve as a terminal respiration system for this trichomonad. The system is capable of carrying out the production of ATP by an oxidative type of phosphorylation. However, the system is not essential for the growth of trichomonads at least under laboratory conditions.

SUMMARY

1. Glucose fermentation balances were performed on Trit. augusta during growth in a continuous culture system and during maintenance in Warburg vessels using standard manometric techniques. Analysis of the medium in both cases showed the same major end products were produced. These were lactate, acetate, succinate, CO_2 and H_2 . The carbon recovery with a glucose substrate in the manometric study was 95% and in the continuous culture system 89%. However, the gas production in both balances was too high in consideration of the proposed metabolic pathway for gas production and the quantity of acetate formed. Speculations on the reasons for this high CO_2 and H_2 evolution are given.

2. A complete formic hydrogenlyase system has been demonstrated in cell homogenates of Trit. augusta. Also formic acid and acetyl phosphate have been found in ether extracted homogenates. The system resembles the phosphorelastic-type observed in Escherichia coli.

3. The enzymes essential to the conversion of pyruvate to succinate (malic enzyme, fumarase and succinic dehydrogenase) and the intermediates involved (malate and fumarate) have been detected in cell homogenates of Trit. augusta.

To demonstrate fumarase it was necessary to utilize a coupled reaction incorporating an external source of malic dehydrogenase. Electrons were transferred from phenazine metho-

sulphate to a tetrazolium salt. The succinic dehydrogenase of Trit. augusta seems to resemble the mammalian enzyme and not the fumarate reductase described in the anaerobic micrococcus M. lactilyticus.

4. Besides the lactic dehydrogenase system a second type of oxidation mechanism was detected in Trit. augusta which may represent a means for terminal respiration in the trichomonads as well as a mechanism for ATP formation. The importance of this pathway is discussed.

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