GLUCOSE UTILIZATION AND RELATED METABOLISM OF *GIARDIA DUODENALIS* TROPHOZOITES

by

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STATEMENT OF THE PROBLEM

The recent cultivation of certain Giardia spp. trophozoites in pure culture (1) (136) has made it possible to begin the physiological study of these organisms. It is the purpose of this research to investigate some of the basic metabolic characteristics of Giardia duodenalis. The ultimate objectives of such a study are to determine what compounds can be utilized for energy production and as carbon sources and what metabolic mechanisms are involved in the utilization of these compounds.

As this is the first metabolic study of any member of this genus of protozoa, several properties will be investigated. Some of these properties to be determined include: endogenous oxygen uptake by glucose, end products of glucose metabolism, effects of metabolic inhibitors on glucose metabolism, stimulation of ATP production by adding glucose, and presence or absence of cytochromes.

INTRODUCTION

I. THE ORGANISM

A. History

Giardia was probably the first genus of parasitic protozoa to be seen by man. Antony van Leeuwenhoek described, from his own stool, what were likely giardia in 1681.

> . . . I have sometimes also seen animalcules amoving very prettily; some of them a bit bigger, others a bit smaller that a blood-globule . . . Their bodies were somewhat longer than broad, and their belly, which was flatlike, furnisht with sundry little paws, wherewith they made such a stir in the clear medium and amoung the globules . . ; and albeit they made a quick motion with their paws, yet for all they made but slow progress. (2)

Dobell (2), after a study of van Leeuwenhoek's writings, concluded that this rather graphic account is in all probability a description of the size, shape, and particularly the swimming motion, of giardia trophozoites. However, giardia were not fully described until 1850 by Lambl (3,4).

Until 1960 giardia were the only intestinal protozoa that had not been cultured in vitro. In that year Karapetyn (5) reported he had succeeded in growing several species of giardia monoxenically in the presence of viable yeast.

Although he claimed to have grown giardia from man (G. lamblia) and rabbit (G. duodenalis), there are no subsequent reports confirming this work. By modifying Karapetyan's procedures, Meyer

and Pope were able to culture rabbit giardia monoxenically (6) and Meyer was later able to establish axenic cultures of giardia from the rabbit (G. duodenalis), cat (G. felis). and chinchilla (G. chinchillae)(1). Meyer has also recently cultured G. lamblia axenically (82 ,136).

B. Classification and nomenclature

The protozoan genus Giardia is classified as follows: Kingdom Protista, Phylum Protozoa, Class Zoomastigophorea, Order Diplomonidorida, Family Hexamitidae, Genus Giardia (167). Difficulty has arisen in assigning species names to giardia because these flagellates are found in many different hosts (7,8) and because many are indistinguishable morphologically. Earlier workers based species names on the host in which they were found (9,10). Later studies, however, have shown that some species are not always strictly host specific (3,7,14).

Some authorities have tried to differentiate Giardia species by average body measurements of trophozoites. However, in observing cultures of giardia, one can see obvious differences in body size of the same species, depending on age of culture and growth conditions. Indeed, Filice, in an extensive morphologic study of giardia, showed that there is no morphologic means of differentiating many of the so-called species. He concluded that on the basis of morphology only three species can be recognized (11). These include G. muris from the mouse and G. agilis from the tadpole; the rest of the species are placed in a third group, G. duodenalis.

The "species" in this third group are, in practice, still distinguished from each other for the most part, according to the host which they inhabit: For example, G. caviae from the guinea pig, G. canis from the dog and G. cati or G. felis from the cat. For purposes of this thesis the designation Giardia duodenalis will be used as the name for the organism studied. This organism was orginally isolated from a cat.

The present status of the Giardia species question will probably remain unresolved until more physiologic, genetic, and antigenic data are available about these organisms.

C. Morphology and life cycle

Giardia exist in two forms: as trophozoites which dwell attached to the wall of the duodenum of their host, and as cysts which form from the trophozoite and are passed in the feces (see figure 1). Ingested cysts exist in the host duodenum and give rise to trophozoites which divide by binary fission.

The following describes G. lamblia, a typical species:

> The trophozoite . . . is a bilaterally symmetrical pearshaped flagellate with a broad rounded anterior and a tapering posterior extremity. The usual length is from 9 to 12 μ, width from 6 to 12 μ, and thickness from 2 to 4 μ. The dorsal surface is convex and the ventral flat. An ovoid concavity with raised margins, the sucking disk, occupies about three quarters of the ventral surface. Its anterior edge blends smoothly with the anterior curve of the body, but its posterior edge is indented by a deep notch. The finely granular cytoplasm, free of inclusions, is enclosed in a transparent lamellar membrane, which under the electron microscope has an irregular rough surface.
>
> There are four pairs of flagella. Under the electron

> microscope the flagella are of equal thickness throughout and are transversely striated. The axonemes of the two anterior flagella pass forward from the lateral blepharoplasts, cross each other, and follow the anterior margin of the body to emerge on each side. The axonemes of the two posterior lateral flagella arise somewhere from the blepharoplasts and pass along the margin of the notch of the sucking disk to emerge on each side. The axonemes of the posterior flagella run parallel from the median blepharoplasts along the mid-axis to emerge at the pointed posterior end. The short axonemes of the ventral flagella arise from blepharoplasts in the center of the sucking disk. A fused pair of deeply staining curved bars, considered by some to be parabasal bodies, lies posterior to the sucking disk. These bars of unknown function have been renamed median bodies, since the term parabasal bodies is considered inappropriate. The two oval nuclei, connected with the blepharoplasts by rhizoplasts, are in the anterior part of the body dorsal to the sucking disk. Each contains a large central granular karyosome. A centrosome is present at the junction of the rhizoplast and the delicate nuclear membrane.
>
> The cysts . . . are elipsoid bodies with smooth well-defined walls and finely granular cytoplasm. The shrinking of the contents from the wall is a diagnostic feature. The usual length is from 9 to 12 u and the breadth is slightly more that two thirds of the length. The spherical nuclei, which usually range from two to four in the mature cyst, are as a rule in the anterior end. Many structures of the trophozoites are distinguishable in the cyst: the median bodies, ridges of thickened fibrils arranged more or less in parallel pairs with curved ends, probably representing the axonemes of retracted flagella, and the lateral flagella (3).

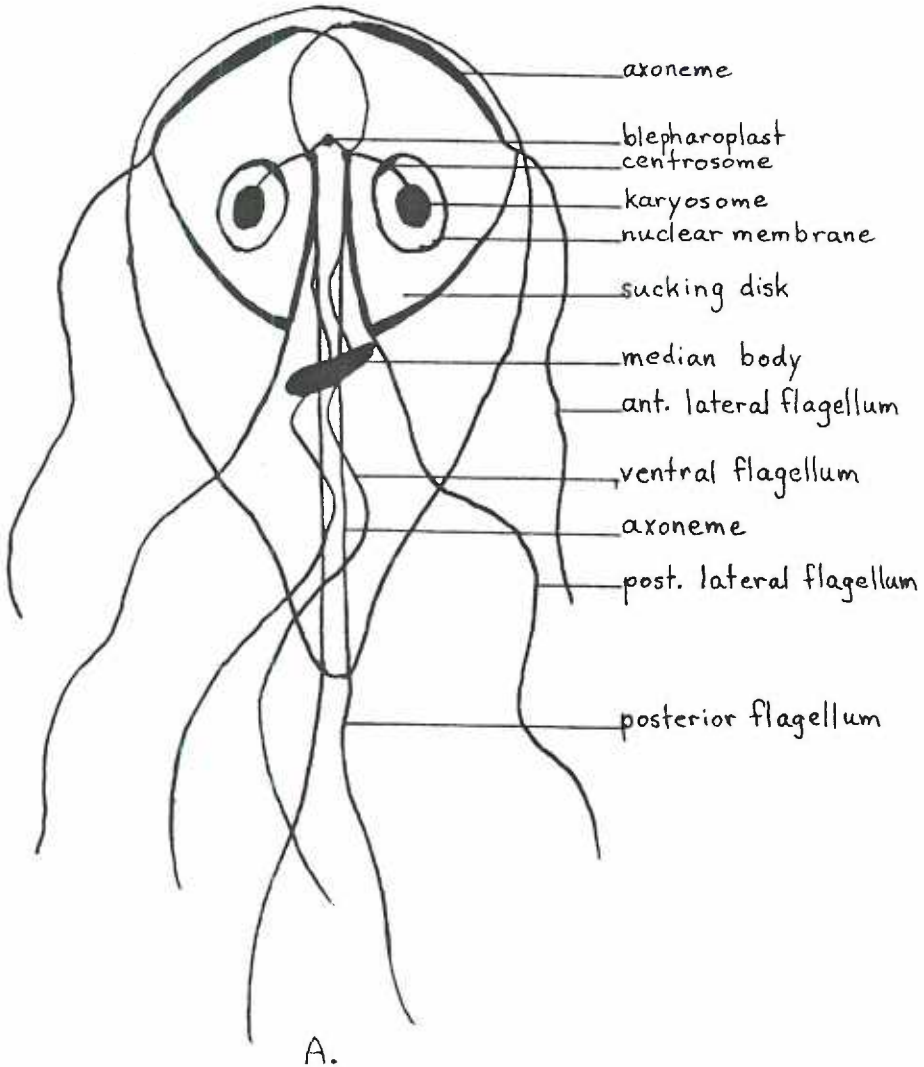
A number of detailed electron microscope studies on Giardia have been published (12,13,161,162,163).

D. Incidence and epidemiology

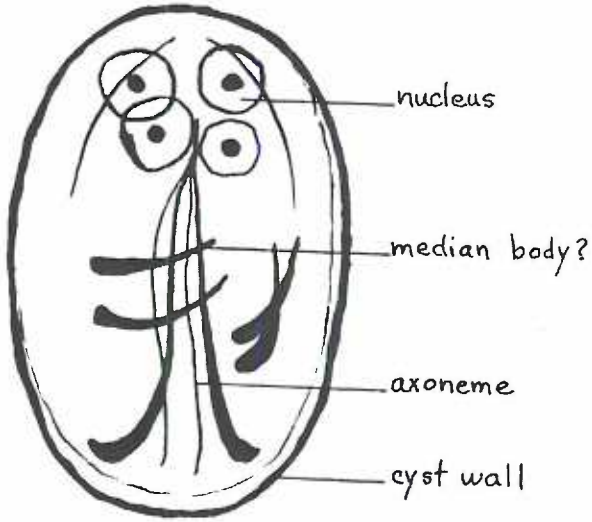
Giardia, common intestinal parasites of a great variety of animals, are widespread in nature (7,8). The human parasite, G. lamblia, has a worldwide distribution (3,4), although it tends to be more common in warm climates (4). Incidence of infection

Figure 1

Giardia lamblia. A. ventral view of trophozoite. B. cyst. After Chandler and Read (84).


axoneme
blepharoplast
centrosome
karyosome
nuclear membrane
sucking disk
median body
ant. lateral flagellum
ventral flagellum
axoneme
post. lateral flagellum
posterior flagellum

A.


nucleus
median body?
axoneme
cyst wall

B.

may vary from as low as 5 or 6 percent (3,15,16) to as high as 100 percent in certain age groups of some populations (4). Children tend to be infected more often than adults and crowded conditions favor the spread of the organism (3).

Although no epidemiological studies have proven the mode of the transmission of the parasite, the evidence indicates, and most authorities agree, that transmission is by food, water, or soil contaminated with cysts, or by person to person contact (3,4,16,17).

E. Pathology

Some individuals may harbor small numbers of G. lamblia for years and never be affected by their presence (3,4,7). In other people, however, large numbers of these organisms cause a disease characterized by persistant or recurring diarrhea. Symptoms often resemble celiac disease, sprue, or steatorrhea with large amounts of unabsorbed fats and/or yellow mucus in the stools. Other common symptoms include epigastric pain, flatulence, anorexia, constipation, apathy, and nervousness (3,4,15,16).

One investigator succeeded in infecting himself with trophozoites obtained by duodenal drainage of an infected person. The following is his account of the experiment:

> In the weeks before April 6, 1966, I intubated myself duodenally six times. None of the aspirates contained protozoa. On April 6, 1966, I inserted the duodenal tube and injected about 10 cc of fluid containing several thousand Giardia trophozoites (source: Oscar Mora).

Up to that time I had had normal bowel movements.

Two days later I had the first feeling of malaise. Several times a day I had the feeling that I was about to vomit. Large amounts of gastric fluid would be "burped up" and swallowed again. This sometimes happened in the middle of the night. In another 24-28 hours, I had diarrhea. The symptoms I had included the following:

(1) Diarrhea - 6 to 8 times a day - until you feel there is no more to give. Contrary to some reports, constipation is not a symptom. Bowel movements are not mushy or loose but frankly watery.

(2) A general feeling of malaise; you feel lousy, just lousy.

(3) Loss of appetite: food doesn't taste good.

(4) Generalized abdominal pains; not in the upper left quadrant but general.

(5) A feeling of fullness in the whole abdominal area, probably the result of the production of much gas; much "burping".

(6) Temperature normal or slightly above; never above 100 F.

My ability to work was cut down. It takes a real effort to do minor tasks. I can feel my heart beating in my head.

My normal weight is 180-181. On April 10th it was 175. On April 13th it was about 170.

(Liquid) stool examination on April 13th yielded many Giardia cysts. Intubation duodenally (on April 13th, about 4 hours after breakfast) was the most difficult ever with a great tendency to vomit. No viable Giardia trophs could be found, although there seemed to be dead cysts and trophs present. Many epithelial cells and some villi were seen. "Lo-Motil" seems to help.

Another duodenal intubation on the morning of April 14th - before breakfast - lasted several hours and yielded 4 test tubes full of fluid - all clear and colorless and devoid of Giardia. Conclude: tube didn't get into the duodenum.

Acranil (0.1 gm per tablet, 3 tablets daily) started on April 14th. Some improvements noted same day; in 2 days sense of well being had returned, and symptoms were gone, as well as the Giardia. Feeling of weakness takes a week or so to abate. (18)

Clinical studies have also shown that malabsorption of fats, fat-soluble vitamins and carbohydrates occurs in many cases (19,20,21,22). Additional clinical studies indicate the

occurrence of physical changes in the epithelium of the duodenum, exfoliation (15) and mucosal defects and abnormalities (4,16,19,104).

Symptoms and pathologic manifestations invariably disappear upon eradication of the parasite (19,20,21). About 90 per cent of giardial infections in humans can be cured easily with quinacrine (Atabrine), chloroquine, acranil, amodiaquin (Camoquin) or metronidazole (3,4,8). It is not known why the other 10 per cent of those infected are apparently refractory to treatment; prompt reinfection or immune deficiencies are possibilities.

F. Culture characteristics

A chemically defined medium for the axenic culture of giardia trophozoites has not yet been devised. The trophozoites presently are grown in a complex fluid medium with a physiologic salt solution as the base. Some component or components of serum appear to be required; the organisms cannot be maintained in its absence. In addition, the presence of certain protein hydrolysates are necessary for optimal growth. The organisms appear to grow best in a pH range of 6.4 to 6.8. The medium must have a redox potential in the range of -70 to -100 mv, which is achieved by the addition of a reducing substance. Since exposure to air over a period of time kills giardia, culture tubes are filled almost to the top before closure with rubber stoppers. Culture tubes are incubated on their sides at 37 C.

Giardia trophozoites have an adhesive disc with which they

attach, in a monolayer, to the glass in culture. Few organisms are seen swimming freely in the medium. Therefore, trophozoites are routinely counted by microscopic observation of organisms attached to the walls of the culture tubes (68).

More detailed information on culture methods is presented in the Methods section.

II. RATIONALE FOR STUDYING THE PHYSIOLOGY OF GIARDIA SPP.

There are a number of reasons for the physiologic study of giardia. Almost nothing is presently known of their physiology. Most parasitic protozoa of importance have been or are being studied physiologically (23,24). Such a study of giardia may lead to a greater understanding of the mechanisms of disease production and the host-parasite interaction.

Giardia in man are a health problem in many parts of the world (3,4,16). It is not known why some infected people develop disease symptoms while others harbor the organisms for years with few if any symptoms. Likewise it is not known why a small percentage (10 per cent) of infected people are not aided by the chemotherapy that is effective in most people. An understanding of the nutrition and biochemistry of these protozoa may aid in understanding how they cause disease and hopefully how it can be prevented.

As stated so well by Wellerson, et al (36) "The search

for an effective chemotherapeutic agent depends entirely on the existence of a difference in the metabolisms of the host and the parasite. Discovery of specific differences in the physiologies of the host and the parasite will provide information essential to the ultimate solution of the problem." This has been borne out in practice. For example: certain trypanosomes which cause disease in humans have been found to have a terminal electron transport system that is completely different from any system known in mammals. This has been used as a target for the development of chemotherapeutic agents(111).

The discovery of special subcellular particles (hydrogenosomes) in trichomonads has given insight into the mode of action of the anti-trichomonad drug metronidazole (118). This resulted from years of study of the physiology of trichomonads. In this case the drug was found effective empirically. Now that the mode of action is more clearly understood, however, it could be used as a tool in elucidating the physiology of other anaerobic parasites.

Metabolic studies of giardia may eventually help solve some of the classification problems of organisms in this genus. Although many species of vertebrates are parasitized by giardia, the present practice of assigning species status to those protozoa according to host specificity or by measurement of the trophozoites has been conflicting and unconvincing. Metabolic

and biochemical data on giardia from various hosts may help in defining their relatedness much as the biochemical characteristics of bacteria are used to establish species relationships. Such biochemical studies may show that the many Giardia spp. presently considered to be species are only different strains of perhaps a few species.

Studies on subcellular particles of different species of trichomonads have indicated that these species may be more closely related than heretofor supposed (151). Such studies may also prove important in elucidating the evolution of metabolic systems in protozoa. Comparative studies on the terminal respiratory systems of trypanosomes have been done in order to determine their evolutionary states (63).

As has been the case with a number of other protozoa, giardia may prove of value in screening chemotherapeutic, chemical and carcinogenic agents and in assays of nutrients (25,26,27). In vitro cultures of giardia have already been used in preliminary evaluations of anti-giardia agents (28).

Moreover, certain protozoa such as tetrahymena (25,29,30), euglenae (31,32,33), amoebae (34,35), trichomonads (36,37,38, 39,40,41), and trypanosomes (42,43,44,45) have been shown to have many enzyme systems in common with higher organisms. They therefore could serve as model systems for metabolic study as

they are often easy to grow, larger than bacteria, easier to break up and are closer, phylogenetically, to higher organisms than bacteria. Giardia, if shown to have similar metabolic systems, could serve as a similar model system.

Since they are parasites that are now relatively easily grown in pure culture, giardia may also serve as a model for investigating a variety of problems of host-parasite relationships.

III. STUDY TO BE UNDERTAKEN

The reasons given for studying this protozoan require some knowledge of the organisms's metabolism. This thesis is concerned with answering the following questions:

A. Do G. duodenalis trophozoites consume oxygen?

B. Do G. duodenalis trophozoites use glucose?

C. Is G. duodenalis growth stimulated by glucose?

D. Is their oxygen uptake stimulated by glucose?

E. What are the end products of the glucose metabolism of giardia?

F. Does glucose affect the level of ATP in giardia?

   1. aerobically?

   2. anaerobically?

G. Do giardia have a glycolytic pathway?

H. Do giardia contain cytochromes?

I. Can giardia oxidize NADH?

J. What effect, if any, do metabolic inhibitors have on giardia?

IV. PHYSIOLOGY OF RELATED ORGANISMS

A. General

A review of all the available literature on the physiology of parasitic protozoa, or even parasitic flagellated protozoa, would require more space than is available here. Therefore only pertinent aspects of the metabolism of related protozoa will be discussed. Since flagellated parasites in the genus Trichomonas appear to be the protozoa most closely related to Giardia both physiologically and phylogenetically, they will be considered here in greatest detail.

B. Utilization of glucose by parasitic protozoa

Many parasitic protozoa are capable of utilizing glucose. Glucose has been found to stimulate the production of CO_2, H_2, acetic acid, ethanol (34,46), lactic acid (47) and to disappear from the medium when given to Endamoeba histolytica (34). Glucose was shown to be consumed and to stimulate O_2 consumption by Leishmania braziliensis (48). Glucose is necessary for the in vitro growth of several malarial parasites and was shown to be metabolized by Plasmodium berghei (49). Likewise, glucose is essential for the in vitro survival of Trypanosoma rhodensiense. The organism produces pyruvate, glycerol and minor amounts of

succinate and CO_2 aerobically from glucose (42). Most, if not all, trypanosomes studied are stimulated to take up O_2 by glucose and to metabolize it to such end products as succinate, lactate, pyruvate, CO_2, and H_2 (23,36,38,40,41,51,52,53,54,55).

C. Metabolic systems in parasitic protozoa

Most of the major metabolic systems found in higher organisms have also been found partly or totally in parasitic protozoa. For example, all of the enzymes of the Emden-Meyerhof-Parnas (EMP) scheme of glycolysis have been found in the Laredo strain of E. histolytica (34) and most of them were found in another strain of E. histolytica by different workers (35). In addition, by giving E. histolytica glucose radioactively labeled in various positions, it was found that the pentose shunt was probably not used, although typical glycolysis occurred (34,56). Since E. histolytica must be grown under near anaerobiasis (47,57), the Krebs (TCA) cycle probably does not function as such in that system. However, as the organism is capable of making succinate, it may contain some TCA cycle enzymes (58). Other evidence suggests that the Entner-Doudoroff pathway may function in certain strains of E. histolytica (35).

Labeling experiments with the blood stream form of T. rhodesiense indicate the presence of the EMP pathway of glucose breakdown (42). Further, several TCA cycle enzymes have been found in both the blood stream and culture forms of T. rhodesiense (59). However, as pyruvate appears to be a main breakdown product of glucose in this organism, Grant and Fulton argue that the cycle may not function as such (42).

T. cruzi has been shown to have some TCA cycle enzymes (59) as well as some of the intermediates of the pathway (60). This organism can also oxidize intermediates of the TCA cycle (45). In addition there is evidence that the parasite utilizes the pentose phosphate (pentose shunt) pathway (43).

Various glycolytic enzymes and phosphorylated intermediates have been found in most of the species of trypanosomes studied metabolically (60,61,62). As for terminal respiratory systems in trypanosomes, Fulton and Spooner found cytochrome systems in the insect and blood forms of the T. lewisi group and in the insect form of T. gambiense; no such system could be found in the T. vivax, congolense or brucei groups (63).

It has been found that those trypanosomes with cyanide sensitive aerobic metabolism characteristically have conventional glycolytic and TCA pathways. Reduced dinucleotides are oxidized along with succinate by flavoproteins which are linked to hemoproteins and oxygen. The hemoproteins resemble mammialian cytochromes, but are evidently not directly comparable to them (113,114,138). In trypanosomes which are insensitive to cyanide, glycolysis occurs but the TCA cycle is inactive. The NADH oxidizing system in these organisms appears to be a coupled reaction of two enzymes, an L α glycerophosphate oxidase with an NAD-dependent dehydrogenase. The system is particulate and it is likely that it is contained in a subcellular organelle called an α-glycerophosphate oxidase body (105,112,113,114,123).

Marr (106) considers the ability of trypanosomes to use intermediate products of metabolism as hydrogen acceptors an adaptive measure lacking in most eucaryotes. This may well be true of most parasitic protozoa.

TCA cycle enzymes and metabolic intermediates occur in several species of Leishmania (48).

Of the trichomonad species studied, the metabolic systems of T. vaginalis have been most studied. Several workers have shown that T. vaginalis apparently follows the EMP scheme of glycolysis. Enzymes of the pathway as well as phosphorylated glycolytic intermediates have been found (39,40). The action of several metabolic inhibitors on the respiration of T. vaginalis points to a functioning EMP pathway in this organism (41). Several other species of trichomonads (T. foetus, T. gallinarum, and T. suis) have been shown to contain glycolytic enzymes (38).

Evidence for the existence of the TCA cycle in most trichomonads is lacking or contradictory. Most Trichomonas species cannot oxidize intermediates of the cycle (41,64); conflicting reports exist for T. gallinae (65,115). T. vaginalis may or may not do so; Tsukahara has shown that the organism can oxidize certain of the intermediates, while other workers have not been able to demonstrate this (64,65). Of the TCA cycle enzymes, only malic dehydrogenase has been definitely demonstrated in trichomonads (38,59,64). Isotopic tracer studies with labeled

pyruvate showed no incorporation of label into TCA cycle intermediates in T. vaginalis (64), nor did TCA cycle inhibitors affect this organism's oxidative activity (64).

Cytochrome systems in general have not been found in trichomonads (36,41,66). Cytochrome b, however, has been shown to be present in T. foetus (41).

Despite the apparent lack of a TCA cycle and a cytochrome system, and necessity for a low redox potential in their media, most trichomonads are capable of taking up oxygen. It has been shown by Wellerson (36) that the amount of O_2 uptake by T. vaginalis was correlated with the oxidation of NADH: that is, there was a direct relationship between the amount of NADH given a cell-free extract and the amount of O_2 taken up by the extract. The NADH oxidase system of the organism could account for the entire amount of O_2 consumed by it.

Recent research into the respiratory activity of trichomonads has established that certain subcellular structures, previously known as chromatic granules and as paracostal and paraxostylar granules, are the site of much of the cell's energy metabolism. They are considered redox organelles and have been named hydrogenosomes because they are the site of H_2 production in certain of these organisms (123).

The principal function of hydrogenosomes appears to be the

anaerobic conversion of pyruvate to acetate via acetyl CoA, accompanied by substrate level phosphorylation and the production of milecular hydrogen (116,121). The hydrogenosomes are capable, however, of aerobic metabolism. Under aerobic conditions the organelles act as respiratory particles (116,154). They appear to perform many of the redox functions of mitochondria, which trichomonads have never been shown to have. They are not thought to be derived from mitochondria, as their structure is different, and certain enzymes and functions are missing, i.e. TCA enzymes, cytochromes, oxidative phosphorylation, NADH oxidizing activity (121,154).

Acetate is a major end product of trichomonad metabolism under aerobic or anerobic conditions. It is suggested that the pathways are similar under both conditions. The difference lies in the terminal electron acceptor. In aerobiosis it is molecular oxygen; in anaerobiosis it is protons $\rightarrow H_2$ and possibly other compounds (116).

D. Effects of metabolic inhibitors on parasitic protozoa

The use of metabolic inhibitors can often give insight as to the metabolic systems present in an organism or cell. Such inhibitors have been used frequently by workers studying the physiology of parasitic protozoa.

The results of experiments with inhibitors must, of course, be interpreted with care as most are rather non-specific in their action (67). Some of the inhibitors that have been used

in the metabolic study of parasitic protozoa are:

Iodoacetate, a sulphydryl (SH) reagent that inhibits phosphoglyceraldehyde dehydrogenase (glycolysis)

Arsenite, an SH reagent inhibiting alpha ketoglutarate dehydrogenase (TCA cycle)

Cyanide, which inhibits cytochrome c (electron transport system - ETS)

Azide, a cytochrome c inhibitor (ETS)

Fluoroacetate, an inhibitor of the TCA cycle

2,4 dinitrophenol, an uncoupler of oxidative phosphorylation (ETS)

Malonate, which inhibits succinic dehydrogenase (TCA cycle)

Fluoride, an inhibitor of enolase (glycolysis)

8-hydrozyquinolin, a cytochrome b inhibitor (ETS)

Hydroxylamine, a succinic thiokinase inhibitor (TCA cycle)

Carbon monoxide, an inhibitor of cytochrome c (ETS)

Malonate and fluoroacetate inhibit L. braziliensis and other leishmanis (48) indicating, along with other confirming data, the presence of a TCA cycle.

The blood stream forms of Strigomonas oncopelti and T. gambiense were found sensitive to cyanide while T. vivax, T. congolense, T. rhodesiense, T. evansi, T. equinum and T. equiperdum showed little if any sensitivity (63); azide sensitivity was less consistent (63). Carbon monoxide

significantly inhibited only T. lewisi and the culture forms of S. oncopelti, T. gambiense and T. cruzi (63).

Of the trichomonads, T. vaginalis was shown to be sensitive to fluoride and iodoacetate, by glycolytic inhibitors (52), but not to malonate and fluoroacetate, TCA inhibitors (52,64), or to cyanide and azide, ETS inhibitors (52); this strengthens the evidence that this organism possesses an essentially anaerobic metabolism. Glycolytic inhibition was also demonstrated in T. foetus. Cyanide and hydroxylamine also inhibited slightly anaerobically but very little aerobically (38,53,55), again suggesting that the organism relies heavily on glycolysis for its energy. Other species of trichomonads exhibit different responses to the various inhibitors (38,54).

E. General - Interpretation of metabolic data

All of the above information simply shows that the physiology of parasitic protozoa is quiet varied among different genera and species while still possessing the general metabolic outlines of higher organisms. Parasites adapting to individual environmental niches may have lost certain enzyme systems when there was no need or advantage to having them. For example, why should an organism like T. vaginalis need a TCA cycle or conventional ETS when it lives in an essentially anaerobic environment? Conversely, the retention of an ETS in trypanosomes would probably be of advantage as these organisms live in the blood - an environment rich in oxygen.

The fact that an enzyme of a certain pathway cannot be demonstrated or a particular inhibitor of that pathway does not inhibit the organism, does not necessarily prove the absence of the pathway. The techniques used by investigators of parasite physiology have, for the most part, been adapted from studies on bacteria and mammalian cells. The physical differences presented by protozoa may be enough to cause a technique to fail. For example, the cell membrane of the parasite may be impervious to an inhibitor that would ordinarily penetrate a mammalian or bacterial cell. Or perhaps a pathway may not be demonstrated because one enzyme in it may be extremely labile and inactivated by standard methods of cell breakage. The enzyme that catalyzes a certain reaction in the parasite may have a cofactor or trace element necessary for activation different from the bacterial or mammalian enzyme.

The demonstration that a parasite may have a certain enzyme, or even all the enzymes, of a pathway, does not mean that the pathway is functionally significant in the organism. The correlation of different types of data, including tracer studies, utilization and production of intermediates, and other enzyme data, must be made before this can be proven. In many cases, not all of these data are yet available for a certain parasite. It is hoped that this study will provide some of the data needed to characterize the physiology of Giardia.

Finally, it should be noted that much of the work on the physiology of many parasitic protozoa has been done on organisms cultured *in vitro* or carried for many years in animals that are not the natural host. There is the possibility, indeed the probability, that these organisms are quiet different from the ones originally isolated. Adaptation to culture or a different host, or mutation over a period of time, may alter the organism in any of a variety of ways including virulence. The very act of putting a species in culture probably selects for those organisms most suited to survive *in vitro*. These may not represent the largest population of the organism in nature. Or perhaps the different environment *in vitro* allows enzymes, unexpressed *in vivo*, to function or *vice versa*. A facultative organism may use entirely different modes of metabolism aerobically and anaerobically *in vitro*. What does it do *in vivo*? Cultured organisms may very well differ antigenically from those in nature; this might interfere with the use of antigen-antibody reactions to identify strains or species of a parasite. These things must be taken into consideration when studying the protozoan and caution used when interpreting physiologic data.

V. APPROACHES TO THE PROBLEM

The main objective of this research was to examine, by means

of several techniques, the use of glucose by G. duodenalis. Standard Warburg techniques were employed to study the organism's endogenous and glucose-stimulated oxygen uptake, and to study the effects of cyanide on respiration. In addition, the oxygen electrode was used to screen the effect of several other metabolic inhibitors on the uptake of O_2 by the parasite.

Standard chemical tests were used to determine the amount of glucose in the medium, and the amount of protein and reserve polysaccharide (glycogen) in giardia trophozoites. Techniques for ATP, NADH oxidation and cytochrome determinations were also employed.

Radioactive tracer methods were used in conjunction with paper chromatography to study the intermediates and end products of glucose metabolism.

Glucose, both uniformly labeled, and labeled in the 1 and 6 position, was used to study the pathway of glucose degradation.

Growth experiments were employed to determine the effects of varying concentrations of glucose and metabolic inhibitors.

These techniques are described in detail in the Methods sections.

MATERIALS

I. CHEMICALS

The chemicals used in this study are listed here by the company from which they were purchased. Most of the chemicals employed were of analytical reagent grade. If this was not available, the highest grade obtainable was used. Chemicals were stored according to manufacturer's recommendations.

Allied Chemicals, Morristown, N.J.: Sodium fluoride, CP*; acetic acid, R; acetone, AR; potassium tartrate, R.

J. T. Baker Chemical Co., Phillipsburg, N. Y.: Hydrochloric acid, AR; calcium hydroxide, pure.

Baltimore Biological Laboratories, Baltimore, MD.: Phytone peptone.

Coleman and Bell Co., Norwood, Ohio: Xylose, CP; ammonium molybdate, CP.

Commercial Solvents Corp., Agnew, Calif.: Ethanol, 95%.

Department of Clinical Pathlogy, U.O.M.S.: Human serum. Serum was processed in the laboratory by inactivating at 56 C for 30 minutes, sterilizing by Seitz-filtration and storing at -20 C until used.

Difco Laboratories, Detroit, Mich.: Blood agar base; nutrient agar; fluid thioglycollate medium.

Eastman Kodak Company, Rochester, N.Y.: Methyl red; Kodak D-19 developer; Kodak all purpose fix.

*Note: CP indicates "chemically pure" grade, R indicates "reagent" grade, AR indicates "analytical reagent" grade.

Hartmen-Leddon Co. (Harelco) Philadelphiz, Pa.: Folin-Ciocalteu phenol reagent, 2N strength.

Industrial Air Products, Portland, Ore.: Nitrogen, 100 UL-^{14}C-glucose, 6.7 mc/mM; ^{14}C-1-glucose, 10 mc/mM; ^{14}C-6-glucose, 48.7 mc/mM.

Mallinckrodt Chemical Works, St. Louis, Mo.: Magnesium sulfate·7H_2O AR; potassium cyanide, R; magnesium chloride, R; hydrogen peroxide, CP; copper sulfate, R; zinc sulfate·7H_2O, AR; lactic acid, AR; toluene, AR; anhydrous methanol, AR; phenol, AR; perchloric acid, AR; t-butanol, AR; sucrose, AR.

Metheson, Coleman and Bell, Cincinatti, Ohio: Potassium hydrogen phosphate, R; trichloroacetic acid, R; n-butanol, R; ninhydrin; D-fructose, 8-hydrozyquinolin, R; 2-4 dinitrophenol.

Merck and Co., Rahway, N. J.: Magnesium chloride, AR; disodium hydrogen phosphate, R; sodium dihydrogenphosphate, R; sodium citrate, R; barium hydroxide·8H_2O, AR; tartaric acid, CP; calcium chloride, AR.

Nutritional Biochemicals Corp., Cleveland, Ohio: Iodo-acetic acid; alpha amino-n-butyric acid; galactose; gluconic acid; succinic acid; fumarate, monopotassium salt; malic acid; sodium pyruvate; alpha-ketoglutaric acid.

Pfsastiehl Chemical Co., Waukegan, Ill.: Glycogen, CP; maltose, CP.

Pfizer, Co., New York, N. Y.: Penicillin G, potassium; streptomycin sulfate.

Sigma Chemical Co., St. Louis, Mo.: Bovine serum albumin; 2,5-diphenyloxazole; 1,4-bis 2-(5-phenyloxazolyl)-Benzene; Phenyloxazolylphenyl-oxazolyphenyl; sodium tetraborate; boric acid; glucose-6-phosphate, monosodium salt; fructose-6-phosphate, disodium salt; ribose-5-phosphate, disodium salt; 6-phosphogluconic acid, trisodium salt, beta-phenyl-ethyl-amine; ortho-dianisidine; cysteine; leucine; isoleucine; valine; arginine; histidine; methionine; tryptophant; glutamine; serine; threonine, proline; hydroxyproline; asparagine; aspartic acid; cystine; glycine; lysine; alanine; phenylalanine.

Worthington Biochemicals: Glucose oxidase; horseradish peroxidase.

II. INSTRUMENTS & APPARATUS

The instruments and apparatus used in this study are listed here with model numbers, company and address. The materials are listed as nearly as possible in the order in which they appear in the Methods section.

Silicon rubber stoppers, size 0, Bellco Glass Inc., Vineland, N. J.

Culture tubes, 15 ml pyrex, Corning Glass Works, Corning, N. Y.

Binocular microscope, Model SRBL, Swift Instruments, Inc., San Jose, Calif.

Platinum combination redox electrode, model 39186, Beckman Instruments, Fullerton, Calif.

Autoclave, model 8816M, American Sterilizer Col, Erie, Pa.

Bacteriologic filter, 0.2μ, Millipore Corp.

Aspirator bottles, Pyrex, Corning Corp.

Germicidal lamp, model G15T8, General Electric Co., Pleasanton, Calif.

Refrigerated centrifuge, model RC-2, Ivan Sorval Inc., Norwalk, Conn.

White rubber stoppers, size 00, Bellco Glass Co.

Incubator, model 322, National Appliance Co., Portland, Ore.

Glass tubing, 16 mm pyrex, Corning Glass Works.

Stainless steel Morton-type closures, Bellco Glass Co.

Polycarbonate centrifuge bottles, 250 ml, Scientific Products, Evanston, Ill.

Angle type centrifuge head, model GS, Ivan Sorval Inc.

Corpuscle counting chamber, Levy-Hausser type, C. A. Hausser Co., Bluebell, Pa.

Warburg flasks, 17 ml, Scientific Products.

Warburg flasks, 7 ml, American Instrument Co. Inc., Silver Springs, Md.

Warburg apparatus model 66690, Precision Scientific Co., Chicago, Ill.

Oxygen monitor model 55, Yellow Springs Instrument Co., Yellow Springs, Ohio.

Three chart speed recorder, model SRG, Sargent-Welch Scientific Co., Anaheim, Calif.

Water bath model 220, National Appliance Co.

Spectrophotometer, model spectronic 20, Bausch and Lomb Co., Rochester, N. Y.

Cuvettes, Spectronic 20, 6 ml., Bausch and Lomb Co.

Disposable, constricted tissue culture tubes 16 mm I.D., Arthur H. Thomas Co., Philadelphia, Pa.

Filter paper- Whatman #1, W & R Balston Co., England.

Water bath, shaking, Lab-Line Instruments Inc., Melrose Park, Ill.

Scintillation vials, Kimax 20 ml borosilicate, Kimble Products, Toledo, Ohio.

Centrifuge, model HN, International Equipment Co., Needham Height, N. J.

Disposable plastic tubes, 15 ml, Falcon Plastics, Los Angeles, Calif.

Mixer, Vortex Genie model K-550-G, Scientific Industries Inc., Queens Village, N. J.

Scintillation system, model LS 200 B, Beckman Instruments.

X-ray film, 8x10 inch Eastman "No-Screen", Eastman Kodak Co., Rochester, N. Y.

Chromatography sprayer, polypropylene, Bell-Art products, Pequannock, N. J.

Spectrophotometer, recording model 15, Cary Instruments, Monrovia, Calif.

Sonicator, model Biosonic III, Bronwill Scientific Co., Rochester, N. Y.

Spectrophotometer, model DU, Beckman Instruments.

Recorder, optical density, Gilson Electronics.

pHmeter, model 7401-A2, Leeds and Northrup Co., North Wales, Pa.

Fluorescence spectrometer, model 203, Perkin-Elmer Corp., Wilton, Conn.

For routine weighing of chemicals a triple beam Ohaus Centrogram balance was used (Ohaus Scale Corp., Floham Park, N. J.). If four-place accuracy was needed, a type 132368 Sartorius analytical balance was used (Brinkmann Instruments Inc., Westbury, N. J.).

METHODS

I. THE ORGANISM

Giardia have been isolated from the cat, rabbit, and chinchilla (1) and human (136). Gardia from the cat were chosen for this investigation because, of the four strains, they generally grow *in vitro* in highest numbers. The organism used here was originally isolated in monoxenic culture with *Saccharomyces cerevisiae*, in 1965 from an infected cat provided by the Animal Care Department of the U.O.M.S., and has been cultivated axenically since 1966. This study was limited to only one of the above mentioned organisms for several reasons including 1) expense, 2) difficulty in propagation and handling and 3) lack of physical facilities.

II. STOCK CULTURES

Stock cultures of *G. duodenalis* trophozoites were maintained in 15 ml silicon rubber stoppered (Bellco #0) glass culture tubes. Tubes were filled with medium so that less that 2 cm of air space remained. Cultures were initiated by making a 1:10 dilution of fluid from an existing culture with fresh medium. Culture tubes were incubated on their sides in a rack

at 37 C. Periodic checks for bacterial contamination were made by inoculating culture fluid onto nutrient and blood agar media and into fluid thioglycollate medium (all Difco Products). Stock cultures were transferred to new tubes (new stocks made) every 3-4 weeks due to the buildup of a dark insoluble substance on the glass which, although apparently not affecting giardial growth, made viewing and counting the organisms difficult. Old culture fluid was removed and fresh medium added every three days.

Giardia trophozoites have an adhesive disc with which they can attach to glass in vitro; since the organisms grow in a monolayer on the glass, cell numbers are most easily estimated microscopically by a visual scoring system. This method was devised and standardized specifically for giardia cultures. The system is based on counting the number of organisms per low power or high power field and assigning a score to that field (e.g. score 1 = 1 to 10 organisms, score 2 = 10 to 225 organisms). Several fields are counted per tube in a specified manner i.e. specific place in the tube. The scores are added and this value can be used to compare the culture to other cultures counted in the same way.

It is possible to demonstrate multiplication of the organism with this method. An increase in the total score of a culture from day to day indicates multiplication of the organism. Growth curves for giardia have been prepared

correlating this method with an electronic counting technique (colter counter). The visual scoring system is much simpler, quicker and less expensive to use than the electronic method and compares quite well with it (68).

III. CULTURE MEDIUM

A chemically defined medium for Giardia has not yet been developed. The following medium, HSP-1, permits growth of monolayers of trophozoites from an initial 1:10 dilution in about 72 hours. One hundred milliliters of HSP-1 medium contains:

Base:		
Hanks' Balanced Salt Solution (without bicarbonate)	85	ml
BBL Phytone peptone	1	gm
Cysteine HCl	0.1	gm
Additives:		
Human serum (inactivated, Seitz-filtered)	15	ml
Penicillin G	50,000	units
Streptomycin sulfate	0.1	gm

The composition of Hanks' solution and analysis of Phytone will be found in appendices A and B respectively. The Phytone and cysteine are dissolved in the Hanks' solution and autoclaved at 15 p.s.i. for 15-20 minutes. This base is cooled below 50 C and the sterile serum, penicillin and streptomycin added aseptically. The complete medium is added to cultures at a temperature no higher than 37 C. Since oxygen, in the concentration present in air, appears to be toxic for giardia growth

and multiplication, and since the medium must have an oxidation-reduction potential of -70 to -100 mv (measured with a redox electrode) for good giardia growth, this medium must be used shortly after it is prepared. If stored for long periods, oxygen dissolves in it and the redox potential rises. The pH of freshly prepared medium is about 6.4.

IV. MEDIA FOR GROWTH EXPERIMENTS

Media for growth experiments were prepared by addition to or deletion from HSP-1 before or after autoclaving. Substances added after autoclaving were first dissolved in Hanks' solution and the equivalent amount of Hanks' left out of the medium base. These substances were sterilized by filtration (0.2 μ Millipore filter).

V. CULTURING THE ORGANISMS - PHYSICAL MANIPULATIONS

A. Apparatus

Sterile medium to be added to stock cultures for the preparation of large numbers of cultures for harvesting was first poured into a sterile Pyrex aspirator bottle fitted with attachments such that medium could be delivered from it aseptically. All operations involving inoculations and medium changes

were carried out in an isolation hood fitted with a germicidal ultraviolet lamp (General Electric). The aspirator bottle was placed on top of the hood with rubber tubing extending inside the hood; in this way medium could be delivered by gravity flow and the use of large numbers of pipettes was avoided. This saved much time and effort.

If new cultures were being prepared, the protozoan inoculum was added directly to the medium which was then put in the aspirator bottle. Swirling the bottle occasionally while filling tubes ensured a uniform concentration of organisms in all tubes. Aseptic technique was followed at all times.

B. Preparation of new cultures

Parent cultures to be used as a source of organisms for new cultures were first placed in a refrigerator (about 4C) for 30 to 45 minutes. This does not affect giardia viability but causes most organisms to detach from the glass and float free in the medium. The tubes were then inverted (not shaken!) ten times, the stopper removed and the contents poured into a sterile vessel of fresh, sterile medium. Organisms to be used for growth experiments were poured directly into sterile centrifuge tubes for washing and concentration.

The stock culture tubes with culture fluid removed still contained many trophozoites attached to the glass; with addition of fresh medium they were subculturable again in two or three days.

C. Preparation of cultures for growth experiments

Certain experiments tested the effect of deletion or addition of some substance to or from the medium on the growth and multiplication of giardia. Trophozoites used in such experiments were first washed by centrifugation in sterile Hanks' solution. The concentrated cells were resuspended in an amount of test medium to yield the desired concentration and dispensed into 6 ml tubes which were then stoppered with #00 white rubber stoppers (Bellco). Each culture tube was incubated on its side in a rack at 37C. Daily microscopic observations were made of each culture and the growth response recorded.

D. Preparation of large numbers of trophozoites

For experiments in which large numbers of organisms were required, it was found simpler to inoculate several very long rather than many short (15 ml) tubes. These organisms are found predominently attached to the lower surfaces of culture vessels and not free swimming to any extent. Therefore the high surface to volume ratio found in commercially available narrow tubes was chosen for culture vessels. The organisms will grow in a monolayer at least halfway around a tube. Since the culture vessel must be filled almost completely with medium, even low-form Erlenmeyer flasks or ordinary tissue-culture bottles would have been wasteful of medium. Extremely thin, flat bottles with a very large surface area would have been

ideal if they had been available but nothing of this kind could be found. For this reason, ordinary 16 mm Pyrex tubing was cut to three foot lengths (to fit the autoclave). The ends were fire-polished. The empty tubes were autoclaved with one end closed with a size 0 silicon rubber stopper and the other capped with a Bellco stainless steel Morton-type closure. After addition of medium and inoculum the open ends were closed with sterile silicon rubber stoppers, and the tubes incubated on their sides at 37 C. These tubes held 140-150 ml of medium; ten such tubes provided enough trophozoites for most experiments.

VI. HARVESTING ORGANISMS

A. Suspending medium for harvested trophozoites

It was necessary to determine if trophozoites suspended in solutions other than growth medium would remain viable and in good condition long enough to perform certain physiological tests. It was decided that the criterion of viability would be the ability of the organisms to establish themselves in culture after exposure to these suspending agents.

Organisms were centrifuged out of the growth medium in the cold, resuspended in the test solution, centrifuged again and resuspended in fresh test solution. Samples were held at room temperature and aliquots of sample taken after 30, 60,

and 90 minutes. These aliquots were placed in culture medium (HSP-1) and incubated for three days. If the organisms survived and multiplied, the suspending solution was considered satisfactory. THe results of the experiments are shown in table 1.

As Hanks' BSS was a part of the growth medium and always available in large quantities in the laboratory, it was selected as the routinely used washing and suspending solution for future experiments. It should be noted that, ordinarily, organisms used for experiments were kept in an ice bath and not allowed to stand at room temperature. As shown in table 1 propagation of giardia held for 60 and 90 minutes at room temperature was poor.

B. Collecting cells

Most experiments in this study required large numbers of protozoa suspended in Hanks' solution. In such cases, cultures were prepared in the long tubes described in an earlier section. At about 72 hours, the tubes were placed in the cold (4 C) for one and one half hours; this caused most organisms to detach from the glass, and upon inverting the tubes four or five times even more organisms were detached. It was found necessary to keep the organisms cold (0-4 C) from this point until they were used in the experiment.

The contents of all tubes were pooled in a large flask in

TABLE 1

EFFECT OF VARIOUS SUSPENDING MEDIA ON THE ABILITY OF GIARDIA TROPHOZOITES TO MULTIPLY

suspending medium	ability to multiply after 30 min.	60 min.	90 min.
saline	+	±	-
saline with 15% serum	+	±	-
saline with 15% dialysed serum	+	±	-
Hanks' Balanced Salt Solution	+	±	-
0.25 M sucrose	±	-	-
0.50 M sucrose	±	-	-
HSP-1 growth medium	+	±	±

+: Giardia multiplied after 3 days in culture.
-: Number of Giardia fewer or absent after 3 days in culture.
±: Equivocal, some cultures survived, others did not.

an ice bath. This pooled material (usually 1400 to 1500 ml) was then distributed to cold 250 ml polycarbonate screw cap centrifuge bottles and centrifuged in a refrigerated centrifuge (Sorval RC-2 susperspeed) at 4 C in an angle head (Sorval GS) at 1500 rpm for 10 minutes. The giardia-free supernatant was carefully poured off. The protozoa were pooled, resuspended in 50 ml cold Hanks' solution and centrifuged in the cold again. This procedure was repeated once and trophozoites finally resuspended in 14 to 15 ml of cold Hanks' solution without glucose. This yielded a 100x concentration of the starting cell population. The suspension was kept on ice until needed for the experiment - usually immediately. As all experiments requiring this type of suspension were of short duration (two hours or less), no attempt was made to keep the harvesting sequence or the subsequent experiment sterile, but chemical cleanliness was observed throughout.

VII. COUNTING OF GIARDIA TROPHOZOITES

Protozoa for each experiment were counted in a Levy-Hausser corpuscle counting chamber. A small drop of the suspension from a Pasteur pipette was used and the organisms were counted in the standard way (69). Two to four samples of the harvested, concentrated organisms were usually counted. In

most cases one sample would give counts of 150 cells or more. Dilution of the sample, if necessary, was done with Hanks' solution and the dilution factor was incorporated into the formula used to calculate the number of organisms/ml. If the suspension was very dilute, samples were counted until at least 200 organisms had been counted.

With experience it was found unnecessary to kill the protozoa to make a count as they do not move rapidly and a sample can usually be counted in 60 seconds. THe following formula was used to calculate the number of organisms/ml:

$$\frac{(\text{Number of organisms counted})(\text{dilution factor})(4000)(1000)}{\text{Number of squares counted}}$$

VIII. WARBURG TECHNIQUES

Standard Warburg techniques were followed for determining the oxygen consumption of and effects of substrates and inhibitors on giardia (70). Standard 17 ml Warburg flasks were used in some cases; 7 ml micro-Warburg flasks were used when only small amounts (10 ml) of cell suspension were available. If the large flasks were used, the total fluid volume added to the flask was 3 ml; in micro-flasks the total volume was 1 ml. Fluid volumes were always adjusted so that the concentration of cells and suspending medium (Hanks' solution without glucose or bicar-

bonate) was the same in all flasks of a particular experiment, unless these were variables of the experiment.

Cells, in Hanks' solution, were placed in the main compartment of the flask; test solutions were placed in the side arms. Cells were counted for each experiment. The number of cells added to a Warburg flask usually ranged from $2x10^7$ to $5x10^7$ cells/ml. The final number of cells/ml or cells/flask was calculated after taking into consideration dilution with salt solution and substrate.

Flasks were equilibrated for 10 minutes in the Warburg apparatus with shaking; 5 minutes with vents open and 5 minutes with vents closed before starting the experiment. Initial readings were usually taken at 0 time and 10 minutes; at that time substrate or inhibitor was tipped into the main compartment. A longer equilibration period was considered detrimental to the cells. Readings were usually taken at 10-15 minute intervals.

If oxygen consumption was being measured, 0.1 ml of 10 per cent KOH was added to the center well of the flask. A piece of fluted filter paper was added to increase the area for absorption of CO_2.

KCN, when used as an inhibitor of O_2 consumption, was added to the center well in appropriate concentration to maintain the correct concentration of CN^- in the sample solution. If KOH had been used alone, cyanide would have been absorbed by

the base and thus changed the concentrations of CN^- in the sample (71). The following table of concentrations of KCN and KOH was used to prepare CO_2 absorbing fluids:

If conc. of HCN in medium is: (mol/l.)	then the CO_2 absorbing fluid should contain:
10^{-2}	10 ml 2N KCN & 0.2 ml N KOH
10^{-3}	10 ml N KCN & 1.0 ml N KOH
10^{-4}	5 ml N KCN & 5. ml N KOH
10^{-5}	1 ml N KCN & 10. ml N KOH

(71)

A thermobarometric control (Warburg flask with water) accompanied each experiment. Endogenous respiration was checked in every experiment by using cells in Hanks' solution but no substrate or inhibitor. Several experiments also included controls with and without substrate but with dead organisms to determine that the bodies of the organisms did not physically absorb oxygen. When it was found that dead trophozoites did not take up O_2, these controls were deleted from further experiments. All Warburg experiments were performed in duplicate flasks and most experiments were run at least two times.

IX. OXYGEN ELECTRODE

An oxygen monitor (Yellow Springs Inst.) employing a Clark-type oxygen electrode was used in some experiments to detect small and/or rapid changes in oxygen tension in suspensions of Giardia. The monitor was calibrated by setting the

scale to read 100 with an air-saturated Hanks' solution held at 37 C. A reading of 100 is equivalent to 5 μl O_2/ml.

Giardia, suspended in cold Hanks' solution, were added to the sample vial. The number of cells used ranged from $2x10^7$ to $5x10^7$ cells/ml in most cases. The sample was brought to 37 C in a water bath and aerated gently for 45 seconds with a stream of air. The vial was then attached to the oxygen monitor. The sample was held at 37 C and constantly stirred throughout the experiment. Substrates or inhibitors could be introduced to the sample chamber through a port with hypodermic needle and syringe.

Readings were taken either manually by reading the scale every 30-60 seconds during an experiment or automatically by attaching the monitor to a recorder (Sargent model SRG). Most experiments ran from 5 to 15 minutes depending on the condition of the cells.

Cells in samples could be re-aerated one or two times when the supply of oxygen was depleted.

X. GLUCOSE ASSAY - GLUCOSE OXIDASE

In experiments to determine the concentration of glucose in the suspending medium, the glucose oxidase method was used (82). The Nelson-Somogyi (72) method was also tried but was found to be more cumbersome, time consuming and less sensitive that the glucose oxidase method.

Samples were deproteinized by the method of Somogyi (72). To a 2 ml sample, 1 ml of barium hydroxide solution (90 gm $Ba(OH)_2 \cdot 8H_2O$ dissolved in 2000 ml distilled H_2O) was added to stop the metabolism. One ml of zinc sulfate solution (100 gm $ZnSO_4 \cdot 7H_2O$ dissolved in 2000 ml distilled H_2O) was added to complete deproteinization. The mixture was shaken well and either filtered or centrifuged. One and one half ml of the clear solution was taken for the glucose assay.

The oxidase reagent was prepared as follows:

1.0 ml of 1% o-dianisidine in methanol
99.0 ml of 0.01 M phosphate buffer pH 7.0
125.0 mg of glucose oxidase
3.0 mg of horseradish peroxidase

Glucose standards were run with every experiment. Stock glucose solutions were made up in Hanks' solution and stored frozen. The standard solutions used were 0.5,10,25,50 and 100 μgm/ml. For use in the assay, 2 ml of standard were treated with the deproteinizing solution in the same manner as an unknown sample. Of this treated solution, 1.5 ml was taken for assay. The assay was performed as follows:

For each tube, use

1. 1.5 ml standard, unknown or water (for reagent blank)
2. 3.5 ml oxidase reagent
3. Incubate mixture 20 minutes at 37 C
4. Stop reaction with 1 drop of 4 N HCl

5. Read optical density (O.D.) at 400 mu on a spectrophotometer

All unknowns and standards were run in duplicate. Most experiments were repeated two or three times. Samples were read in a Spectronic 20 spectrophotometer against a reagent blank. All samples were read in the same cuvette (Spectronic 20 cuvette) to reduce error as much as possible. Glucose concentrations in unknown samples were calculated by comparing the optical density (O.D.) of the sample with the O.D. of the most similar standard and applying the following formula:

$$\text{Glucose conc. of unknown} = \frac{\text{O.D. of unknown}}{\text{O.D. of standard}} \times \text{Glucose conc. of standard}$$

This technique was used, in most cases, to determine if giardia removed glucose from their suspending medium and if so, at what rate. The assay was also used to check the concentration of samples of radioactive glucose used in other experiments.

XI. PROTEIN ASSAYS

Protein determinations on giardia were carried out using two methods. The Lowry method of protein determination (73) was used most often because of the ease and sensitivity of the method. Several experiments were carried out, however, using a Nesslerization technique (74) to check the accuracy of the

Lowry method (Table 2). Bovine serum albumin (BSA) was used as the standard in all cases. When it was found that the Lowry method and Nesslerization gave the same results, the Lowry determination was used in all subsequent experiments.

For the Lowry determination, a standard curve was prepared using BSA as the standard. The curve extended from 0 to 100 µgm protein (Figure 3). For preparation of the reagents, see Appendix C. The procedure is as follows:

1. Mix well 5 ml of reagent C with 1.0 ml of unknown or standard containing 5 to 100 µgm protein.
2. Add 0.5 ml reagent E rapidly with mixing. Let sit 30 minutes or longer.
3. Read in a colorimeter or spectrophotometer at 750 mµ for 5 to 25 µgm protein/ml or at 500 mµ for more concentrated solutions.

The Biuret protein determination (75) was also tried but it was found that the protozoa would not dissolve in the reagent and therefore gave inaccurate readings.

Figure 2

Standard curve for Nessler's method of protein determination. Micrograms of nitrogen are multiplied by 6.25 to obtain micrograms of protein.

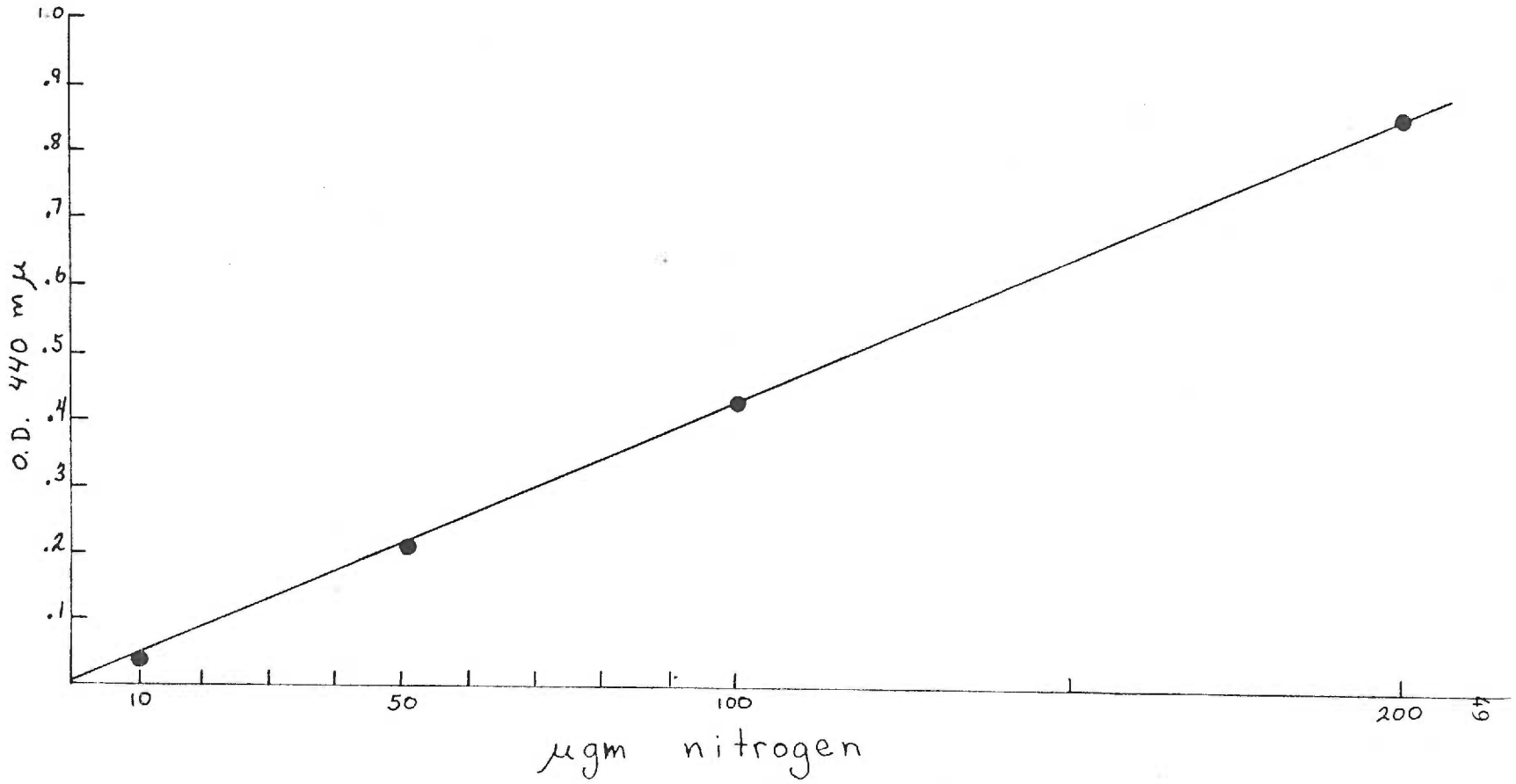

1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
O.D. 440 mμ
10
50
100
200
μgm nitrogen

TABLE 2

Comparison of Lowry and Nesslerization Methods of Protein Determination of Samples of Giardia Trophozoites.

Nessler Measurements (O.D. 440 mμ)

Sample 1	Sample 2
.360	.420
.355	.426
.362	.424
.361	.421
.359	.420
.357	.423
$\bar{X}$ .359 = 512 μgm protein	$\bar{X}$ .422 = 606 μgm protein
S.D. ± .002	S.D. ± .002

μg N are read from standard Nessler Curve (fig.9) and multiplied by 6.25 to obtain μgm protein

Lowry Measurements (O.D. 500 mμ)

Sample 1	Sample 2
.269	.315
.268	.316
.269	.312
.267	.311
.267	.314
.268	.316
$\bar{X}$ .268 = 173 μgm x3 = 519 μgm	$\bar{X}$ .314 = 205 μgm x3 = 615 μgm
S.D. ± .001	S.D. ± .002

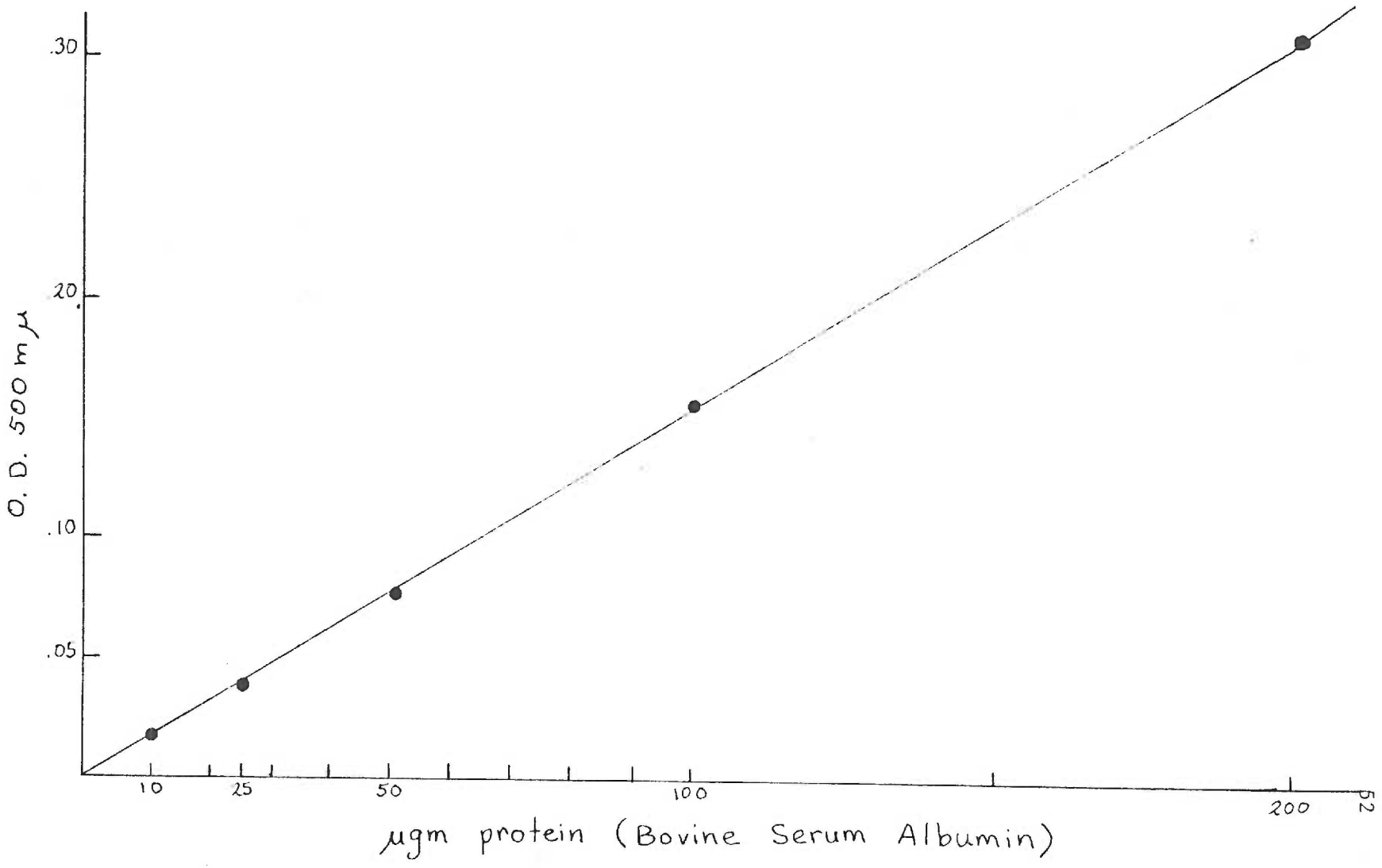
μgm protein are read from Standard Lowry Curve (fig.10) The particular Giardia samples used here had to be diluted 1:3 to be read on the standard curve.

Sample 1 contained 2.8×10^7 organisms/ml

Sample 2 contained 3.9×10^7 organisms/ml

Figure 3

Standard curve for Lowry's method of protein determination.


.30
.20
.10
.05
O. D. 500 mμ
10
25
50
100
200
52
μgm protein (Bovine Serum Albumin)

XII. POLYSACCHARIDE ASSAY

Polysaccharides in G. duodenalis were determined by the method of Montgomery (76). This method is not specific for glycogen but is specific for polysaccharides. It is useful in the range of 10-60 µgm glycogen with a sensitivity to about 5 µgm. The accuracy of the method is ±2.5%. The method is not affected by protein or amino acids and is simple, rapid, inexpensive and reproducible. The reagents consist of concentrated reagent grade sulfuric acid (specific gravity 1.84) and a solution of 20 gm distilled water and 80 gm reagent grade phenol. Purified glycogen was used as a standard. A standard curve was prepared, (Table 3, fig.4) and several standards (0,5,10,25,50 µgm/ml) were run with each set of unknowns, and the amount of glycogen in the unknowns calculated from the formula:

$$\text{Glycogen conc. of unknown} = \frac{\text{O.D. of unknown}}{\text{O.D. of standard}} \times \text{Glycogen conc. of standard}$$

The procedure is as follows: A sample containing 10 to 60 µgm of polysaccharide is diluted with water. In this case, 0.05 ml of a standard 100X concentrated suspension of giardia in Hanks' solution was diluted with 1.9 ml of distilled water. To this was added 0.1 ml of the phenol solution. Following the phenol, 5.0 ml of concentrated sulfuric acid was added in

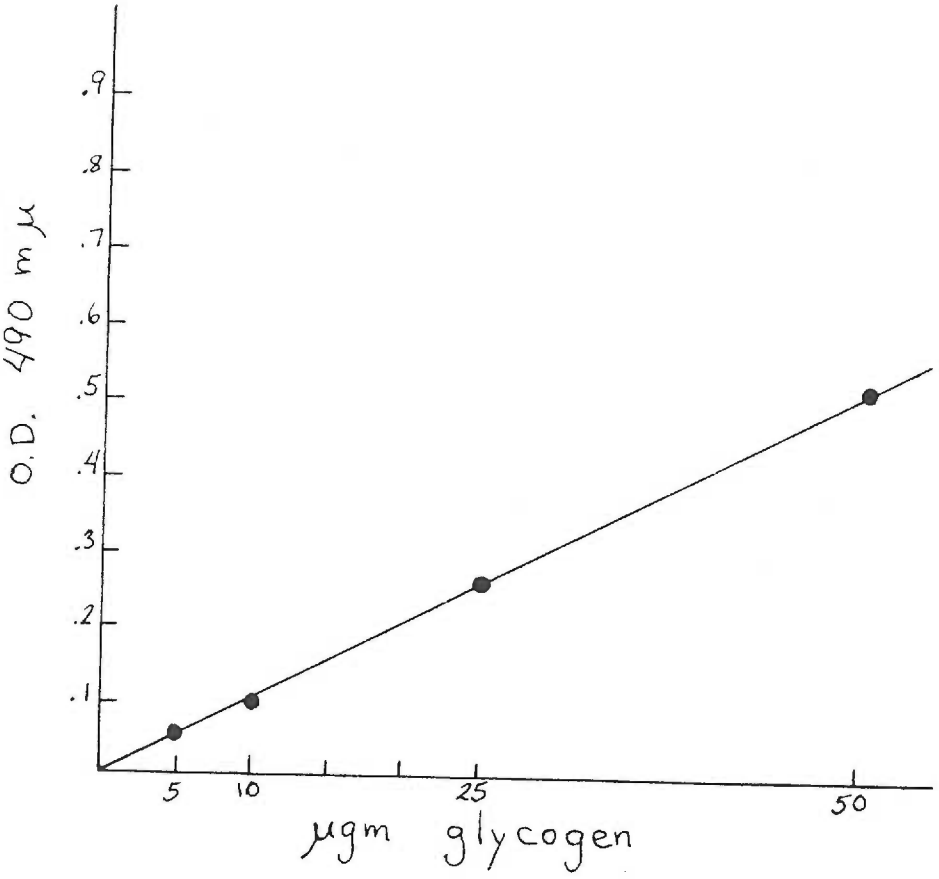
TABLE 3

Glycogen Standards (O.D. 490 mu)

Sample	50 ugm	25 ugm	10 ugm	5 ugm
1	.514	.259	.101	.050
2	.515	.260	.104	.052
3	.512	.258	.100	.051
4	.515	.261	.105	.053
5	.516	.257	.103	.051
6	.517	.259	.103	.053
$\bar{x}$	.515	.259	.103	.052
S.D.	$\pm$ .002	$\pm$ .001	$\pm$ .002	$\pm$ .001

Figure 4

Standard curve for glycogen by the method of Montgomery.


O.D. 490 mμ
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
25
50
μgm glycogen

a rapid stream (using a pipette with a large orifice) against the surface of the liquid in the tube. The heat generated by this addition of acid is necessary for color development. The contents of the tube were mixed thoroughly by inversion. The color (yellow-orange) was read at 490 mμ in the Spectronic 20 after 30 minutes at room temperature. The color is stable for several hours.

XIII. RADIO-LACTATE TECHNIQUE-DETERMINATION OF LACTATE AND CO_2

Because several people handled the glassware and the "fallout" of lactic acid in the laboratory was high, a standard Barker-Summerson (77) type of lactic acid determination was found almost impossible to use. Therefore a modified Barker-Summerson lactic acid assay, determining radioactive lactate in the incubation fluid, was used (78). This assay was developed for use with small quantities of spermatozoa and appeared to be suitable for use with giardia. The method relies on the fact that glucose forms a complex with copper which precipitates when an excess of alkali is added (165). Lactate, however, is not precipitated.

Certain criteria were met to assure the applicability of this method to giardia:

a. Only ^{14}C-lactate was being counted - samples were chromatographed after the copper-alkali treatment. The only labeled compound found was lactate:

b. Proportionality was obtained between giardia concentration and lactate formation (fig.5)

c. The procedure could be replicated (see table 16)

Because of the manner in which the experiments were set up, it was not only possible to determine the production of lactic acid from glucose but also CO_2 and other metabolic products (see section on autoradiography). For simplicity this method will be called the "radio-lactate" method since radioactive lactate was being measured.

A special incubation tube was devised which could be disposed of as radioactive waste after one use (see fig.6). This obviated the necessity of using Warburg vessels as orginally described by Hoskins (78) and aided in keeping the laboratory free of radioactive contaminants. Disposable constricted glass tissue culture tubes, 16mm I.D. were cut to about 10 cm in length. A 30 mm piece of Pyrex tubing, 12 mm O.D., that would rest on the constriction of the tissue culture tube was fitted with a piece of Whatman #1 filter paper that would take up to 0.1 ml of fluid (approximately 3 cm x 2 cm). This piece of tubing constituted the CO_2 trap. To insure no leakage of fluid from the trap, a slight stricture was made at

Figure 5

Correlation of number of organisms with the amount of lactate formed. Radio-lactate method.

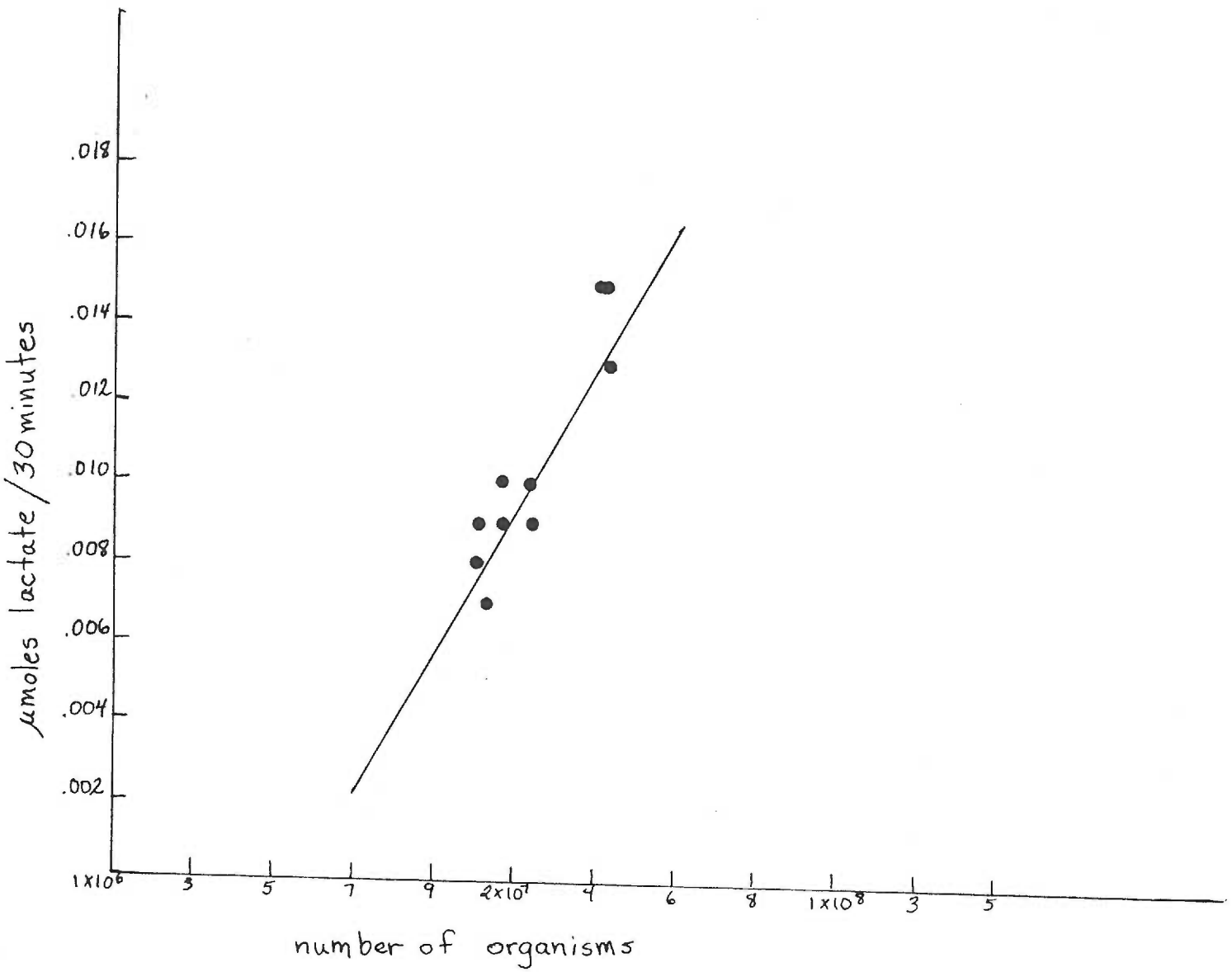
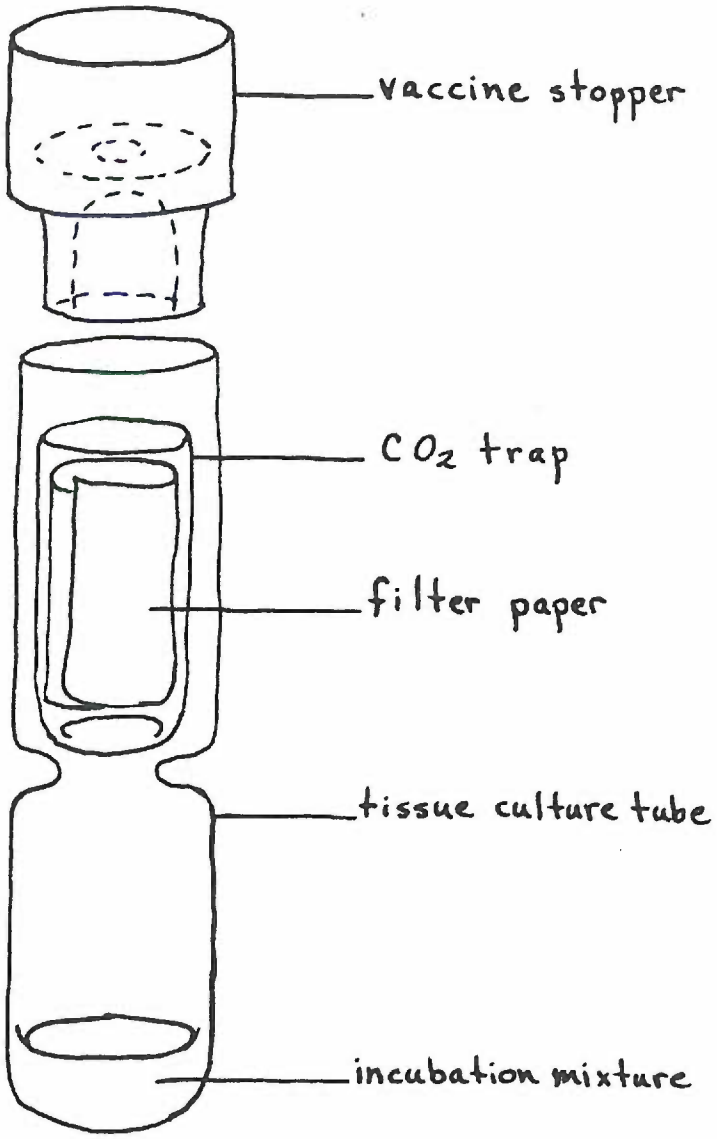

μmoles lactate/30 minutes
.018
.016
.014
.012
.010
.008
.006
.004
.002
1x10^6
3
5
7
9
2x10^7
4
6
8
1x10^8
3
5
number of organisms

Figure 6

Diagram of radio-lactate incubation tube.



radio-lactate incubation tube

the lower end of the tube by melting the end and allowing the glass to collapse inward a bit. The incubation tube was sealed with a number 14 vaccine stopper.

In use, duplicate tubes were placed upright in a rack and the radioactive (^{14}C) glucose in Hanks' solution and any other components of the reaction mixture (except cells) were added to the appropriate tubes. The CO_2 collecting solution (0.1 ml of 1:1 beta phenyl ethyl amine : methanol) was applied to the filter papers in the CO_2 traps. The traps were placed in the incubation tubes and a vaccine stopper fitted on each tube.

At this time, if an anaerobic atmosphere was needed for the experiment, two 18 gauge hypodermic needles were pushed through each stopper and the needles attached to a manifold. The tubes were then flushed for five minutes with nitrogen. If an aerobic atmosphere was desired, no flushing operation was done. The tubes were then placed in an ice bath and cells in Hanks' solution were added through the stopper with a 4 inch 21 gauge hypodermic needle and syringe. Care was taken that the needle touched neither the beta phenyl ethyl amine-saturated paper nor the radioactive fluid in the bottom of the tube.

After cells had been added to appropriate tubes, the entire rack was placed in a shaking water bath at 37 C. In most experiments, the tubes were incubated for 30 minutes. Then

0.1 ml of 25% trichloroacetic acid (TCA) was added to the incubation mixture with a 4 inch 21 gauge hypodermic needle and syringe, care being taken again to avoid touching the filter paper or fluid with the needle. This killed the cells, stopped metabolism and aided in releasing any CO_2 still in solution in the fluid. The tubes were allowed to shake for 30 minutes at 37 C to insure complete removal of CO_2 from the fluid.

At the end of the shaking period, the rack of tubes was removed from the water bath, the stoppers removed and the CO_2 traps carefully removed. Forceps were used to handle the CO_2 traps and filter papers. Each glass trap was carefully rinsed in its own vial of scintillation fluid (see appendix D for preparation) and the corresponding filter paper dropped into the same vial.

The incubation tubes were next centrifuged for 10 minutes at 1500 rpm at room temperature to precipitate the cellular debris. The tubes were removed from the centrifuge and 0.2 ml of each deproteinized solution was placed in a disposable plastic tube, each of which contained 1.3 ml of distilled water and 0.5 ml of a saturated solution of copper sulfate. To this mixture 1.0 gm of calcium hydroxide was added. The plastic tube was then capped and vortexed in a Vortex Genie mixer for 30 seconds every 10 minutes for 30

minutes to insure complete adsorption of all substances except lactic acid. Next, the plastic tubes were centrifuged at 1500 rpm for 20 minutes. One tenth ml of the resulting supernatant was carefully removed to a vial of scintillation fluid (see appendix D for composition).

When all scintillation vials had been filled with CO_2 papers or lactic acid samples, they were counted in a Beckman model LS 200 B liquid scintillation system. Each sample was counted 5 minutes or until the error had reached 0.1%, whichever came first. The scintillation fluids allowed counting at about 80% efficiency.

The amounts (μM) of lactate and CO_2 were calculated in the following manner:

$$\frac{\text{lactate or } CO_2 \text{ sample CPM (counts per minute)} - \text{background CPM}}{\text{CPM due to lactate or } CO_2}$$

all CPMs were corrected for counting efficiency

all CPMs due to lactate were multiplied by 100 in order to compare them directly to CO_2 CPMs. This factor of 100 is used because of the dilution of the incubation mixture during the test for lactate. Therefore:

lactate CPM x 100 = total lactate CPM.

It was found that 1 μM of the UL-glucose used contained 1.11×10^8 CPM. To calculate the μM lactate from lactate CPMs the following formula was used:

$$\frac{1.11 \times 10^8 \text{ CPM}}{1 \text{ } \mu\text{M glucose}} = \frac{\text{total lactate CPM}}{X \text{ } \mu\text{M}}$$

$$X = \frac{(\text{lactate CPM})(\mu\text{M})}{1.11 \times 10^8 \text{ CPM}}$$

$$X = \mu\text{M lactate}$$

since 1 μM glucose → 2 μM lactate
X x 2 = μM lactate produced

For CO_2 calculations no dilution factor was needed. Therefore:

CO_2 Sample CPM
-background CPM
Total CO_2 CPM

and $$\frac{1.11 \times 10^8 \text{ CPM}}{1 \text{ } \mu\text{M glucose}} = \frac{CO_2 \text{ CPM}}{X \text{ } \mu\text{M}}$$

$$X = \mu MCO_2$$

Since 1 μM glucose → $6\mu MCO_2$
X x 6 = μMCO_2 produced.

XIV. CHROMATOGRAPHY AND AUTORADIOGRAPHY

Of the original deproteinized incubation fluid described for the radio-lactate method, 0.3 to 0.5 ml was placed in a small glass tube and dried down with an air stream under a fume hood. The residue was redissolved in 0.1 ml of water or 95% ethanol and 10 to 30 μl were spotted on 8X10 inch pieces of Whatman #1 chromatography paper. The chromatograms were run in the first direction in methonal:ammonium carbonate saturated water (75:25 v/v) and in the second direction in sec-butanol: formic acid:water (85:5:10 v/v).

After the chromatogram was run and dried, it was placed

against an 8X10 inch X-ray film (Eastman "No-Screen"). Manipulations were carried out in a darkroom with a safe-light. The films and chromatograms in paper folders were sandwiched between pieces of 10X12 inch window glass, the sandwiches stacked, and the whole stack wrapped in several layers of aluminum foil and wrapping paper to exclude light. The films were exposed to the chromatograms for two weeks, then developed.

Films were developed in Kodak D-19 developer for 5 minutes at 20 C; if developed at higher temperatures (25-27 C) only 2 to 2½ minutes were needed. Development was stopped in an acid stop bath for 30 seconds and then fixed for about 20 minutes in Kodak all purpose fix. Finally, the films were washed for 30 minutes in running water and dried in a chemical hood in an air stream. This method was adapted from Kamen (79).

Standard chromatograms employing non-radioactive compounds and color reagents were compared with the autoradiograms (see appendix E for chromatographic color reagents). In addition, after the films were developed, some radioactive chromatograms were sprayed with various color reagents in an attempt to identify products of glucose metabolism. THe following is a list of compounds that were chromatographed in the same way as radio-lactate samples and compared with the autoradiograms: Lactic acid, succinate, fumarate, malic acid, pyruvate, alpha keto glutaric acid, citrate, glucose, tartaric acid, alpha amino butyric acid, galactose, gluconic acid, glucose-6-

phosphate, fructose-6-phosphate, 6-phosphogluconic acid, cysteine, leucine, isoleucine, valine, arginine, histidine, methionine, tryptophan, glutamic acid, serine, threonine, proline, hydroxyproline, asparagine, aspartic acid, cystine, glycine, lysine, alanine, phenylalanine and glutamine were chromatographed in one direction in t-butanol:acetic acid: water (70:15:15 v/v).

In some cases, spots on the chromatograms were located by superimposing the corresponding X-ray film on the paper. The spots were marked and cut out. Colored radioactive guide marks were placed on all chromatograms before exposure to X-ray film for this purpose. The spots from the chromatograms were dropped into vials of scintillation fluid and counted. This was a roughly quantitative method of determining the relative amounts of each radioactive compound on the chromatogram.

XV. CYTOCHROME ASSAY

Dr. J. Fellman of the Department of Biochemistry of the U.O.M.S. was kind enough to perform a difference spectrum of a sample of freeze-thaw disrupted Giardia in Hanks' solution. There were 1.3×10^8 organisms/ml in the sample. The sample was reduced with a few crystals of sodium dithionate and the experiment was carried out in a Cary model 15 recording spectrophotometer at

room temperature. The absorption of the sample was observed in the wavelength region of 330 to 700 mμ, which includes the Soret region.

XVI. NADH-OXIDASE ASSAY

This assay was carried out at the Oregon Regional Primate Center in the Laboratory of Dr. D. Hoskins.

Organisms were harvested as described earlier and concentrated to 2 ml in Hanks' solution. THe cells were then sonicated at maximum intensity for 2 minutes in a Bronwill Biosonic III sonicator. The sonicated suspension was centrifuged at 15,000 rpm (28,000 X G) in the RC-2 for 30 minutes. The supernatant was used for the assay.

The assay, according to Hoskins, Whiteley and Mackler (80) is as follows:

In a 1.0 ml cuvette with a 1 cm light path add

Unknown NADH-oxidase (sonicate supernatant)	0.01 ml
0.2M potassium Phosphate buffer pH 6.8	0.2 ml
0.1% NADH	0.1 ml
Distilled water	0.69 ml

NADH oxidizing activity was followed as a decrease in absorbancy (of NADH) at 340 mμ in a Beckman model DU spectrophotometer connected to a Gilson optical density recorder.

XVII. ATP ASSAY

In certain experiments it was desired to know the effect of glucose on the production of adenosine triphosphate (ATP) by giardia.

Organisms were harvested in the usual manner, then incubated in 1 ml volumes in tubes in a shaking water bath at 37 C. Some tubes contained 1 mg of glucose, others had none. Some tubes were aerobic, others had been flushed with nitrogen as described in the radio-lactate method, to obtain anaerobiosis. At 0, 30, 60, and 90 minutes, each of one of the above combinations had 0.1 ml of 60% perchloric acid (PCA) added to kill the protozoa and extract the ATP. The cells were centrifuged out of the incubation fluid at 14,500 rpm for 15 minutes in the RC-2 centrifuge. The supernatants were neutralized with 6N KOH (about 200 µl). A Leeds and Northrup pH meter with a micro-electrode was used to monitor neutralization. The precipitate was centrifuged out at 14,500 rpm for 10 minutes in the RC-2. The supernatants were transported to the laboratory of Dr. Hoskins at the Oregon Regional Primate Center. Here Dr. Hoskins graciously provided the following assay for ATP:

In a cuvette add:

36 mM TEA (triethanolamine) buffer pH 8.0	150 µl
15 mM $MgCl_2$	50 µl
6 mM EDTA	50 µl
60 mM Glucose	100 µl
Unknown ATP (extract of Giardia)	90 µl
Distilled water	50 µl
Hexokinase	5 µl
Glucose-6-phosphate dehydrogenase	5 µl

The reaction mixture was read on a Perkin-Elmer fluorescence spectrometer over a period of several minutes. The amount of ATP in the cuvette was calculated from a standard curve.

RESULTS

I. GROWTH EXPERIMENTS: EFFECTS OF CARBOHYDRATES

A series of experiments were undertaken to determine the effect of adding or deleting carbohydrates from the growth medium. It was soon discovered that these experiments could not be interpreted because of the great variability of results from experiment to experiment. In addition the complexity of the growth medium made these experiments difficult to interpret. Because serum was present one could never be sure that all carbohydrate was deleted from the medium. Dialysed serum could have been used but it could be argued that giardia may possess some enzyme capable of breaking serum polysaccharides down to usable sugars. The Phytone in the medium also contained about 37 per cent carbohydrate. Therefore, experiments involving addition or deletion of carbohydrates would have been difficult to evaluate while the organisms were in this growth medium.

II. GROWTH EXPERIMENTS: EFFECTS OF INHIBITORS

Experiments were set up to determine the effects of cyanide and fluoride on the growth of giardia trophozoites. The results are presented in Tables 4 and 5.

Concentrations of 10^{-2}M through 10^{-4}M cyanide significantly

(P significant at .01 level) inhibited the growth of giardia. Concentrations of 10^{-5}M through 10^{-7}M KCN showed no significant difference from control cultures in Chi square tests (P was not significant at .05 level).

All concentrations of fluoride from 10^{-1}M through 10^{-5}M inhibited the growth of giardia. (P significant at the .01 level). The effect of 10^{-6}M fluoride did not significantly effect the organisms, however, only one experiment with five cultures was performed at this concentration.

TABLE 4

Effect of Cyanide on the Multiplication of Giardia Trophozoites

KCN conc.		test	control	Chi square value
10^{-2}M				
	+	0	6	8.40
	-	15	9	
10^{-3}M				
	+	0	14	43.40
	-	20	6	
10^{-4}M				
	+	2	9	11.73
	-	13	6	
10^{-5}M				
	+	5	7	1.07
	-	5	3	
10^{-6}M				
	+	1	4	2.60
	-	9	6	
10^{-7}M				
	+	0	2	1.88
	-	5	3	

\+ indicates the number of cultures which showed multiplication of organisms 96 hrs after beginning of experiment.

\- indicates the number of cultures which showed no multiplication after 96 hrs.

Chi square values were computed from the formula:

$$Xy^2 = \lesssim \frac{(1f - fc1 - \frac{1}{2})^2}{fc}$$

where y refers to Yates correction for small number of samples (151); f = test = cultures with KCN added, fc = control = cultures with no KCN added.

The level of significance, P, was determined from a table of Chi square values using 1 degree of freedom. P was considered significant at or below the .05 level. In these experiments P was significant at 10^{-2} - 10^{-4}M KCN but not at 10^{-5} - 10^{-7}M KCN.

TABLE 5

Effect of Fluoride on the Multiplication of Giardia Trophozoites

NaF conc.	test	control	Chi square value
10^{-1}M			
+	0	4	15.31
-	5	1	
10^{-2}M			
+	0	7	20.12
-	10	3	
10^{-3}M			
+	1	7	14.40
-	9	3	
10^{-4}M			
+	1	7	14.40
-	9	3	
10^{-5}M			
+	0	7	20.12
-	10	3	
10^{-6}M			
+	0	2	1.88
-	5	3	

\+ indicates number of cultures which showed multiplication of organisms after 96 hrs.

\- indicates number of cultures which showed no multiplication after 96 hrs.

P was significant at or below the .05 level in all cases except at 10^{-6}M F.

III. OXYGEN ELECTRODE EXPERIMENTS

A. Endogenous oxygen consumption

In all oxygen electrode experiments, endogenous giardia respiration was determined by recording oxygen consumption for at least one minute before adding any substrate. In addition, a series of samples was monitored for endogenous oxygen consumption for the full length of time it took the organisms to consume all the oxygen in solution. Table 6 is a compilation of the endogenous respiration of a number of such samples. A scatter plot of these data was made (fig.7). As with the Warburg data there is a wide scatter of points. When the values were normalized to $\mu l\ O_2$ uptake/hr/10^8 cells they fit a normal distribution fairly well[1] (see Table 6). A correlation coefficient of 0.44 was obtained when numbers of organisms were compared to the amount of oxygen they consumed. This low correlation will be discussed later with the corresponding Warburg data.

[1]Many values in succeeding experiments are normalized to 10^8 organisms so more direct comparisons can be made from sample to sample and experiment to experiment.

TABLE 6

Endogenous Oxygen Consumption of Giardia Trophozoites in Hanks' BSS: Oxygen Electrode Measurements

organisms/ml	µl O_2/hr/ml	µl O_2/hr/10^8 organisms
$3.9X10^7$	20	51
$6.6X10^7$	58	88
$5.0X10^7$	49	98
$3.9X10^7$	57	146
$1.3X10^7$	21	162
$3.1X10^7$	53	169
$2.1X10^7$	37	177
$2.7X10^7$	49	182
$2.2X10^7$	41	185
$3.9X10^7$	80	205
$4.5X10^7$	99	219
$2.8X10^7$	65	230
$3.7X10^7$	86	233
$2.0X10^7$	53	267
		mean 172
		S.D.± 58

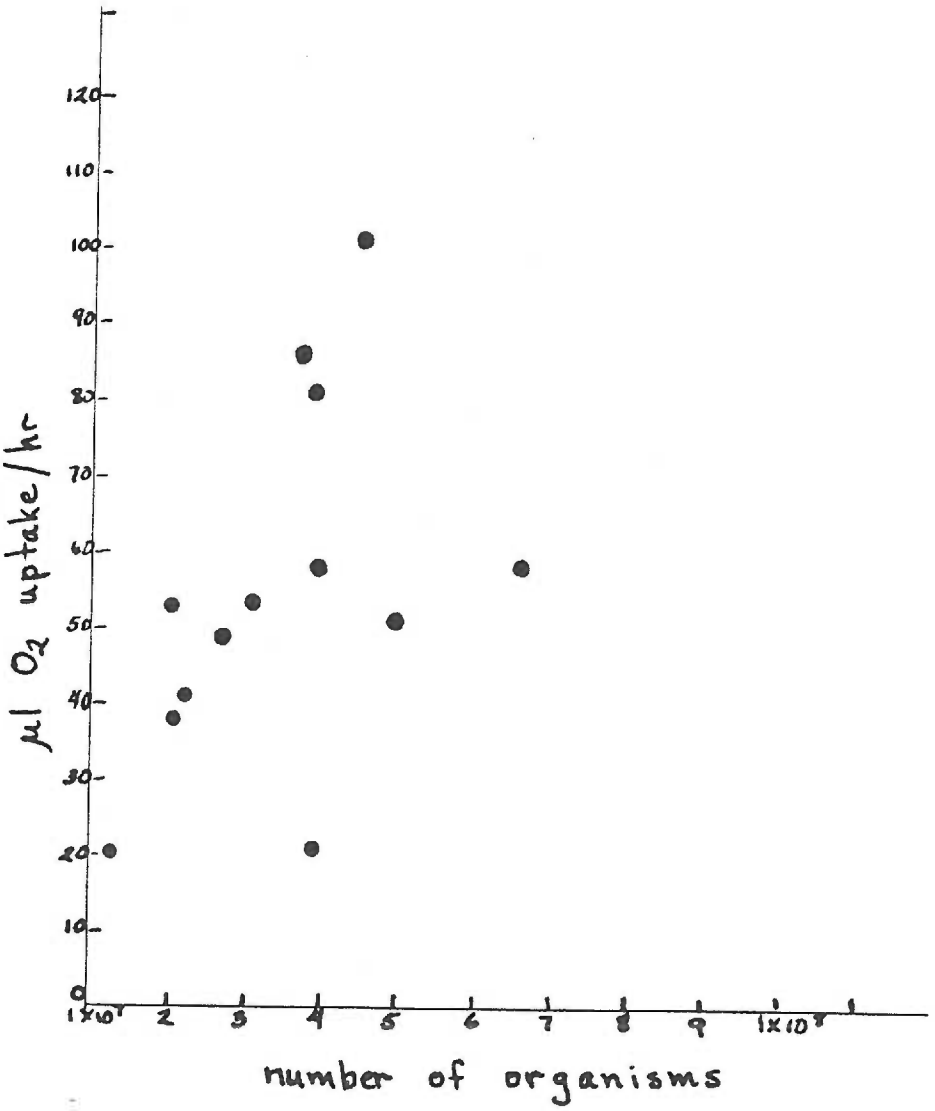
71% of the values fell within ±1 standard deviation of the mean

92% of the values fell within ±2 S.D.s of the mean

100% of the values fell within ±3 S.D.s of the mean

Figure 7

Correlation of number of organisms with the amount of endogenous oxygen consumed in Hanks' BSS: Oxygen electrode measurements.


μl O2 uptake/hr
120
110
100
90
80
70
60
50
40
30
20
10
0
1x10
2
3
4
5
6
7
8
9
1x10
number of organisms

B. Effects of metabolic inhibitors on oxygen comsumption of *Giardia* Trophozoites: Oxygen electrode measurements

Several metabolic inhibitors were screened to determine their effects on the oxygen consumption of trophozoites. Table 7 summarized these data. Only cyanide was studied to a great enough extent to provide enough samples to analyze statistically. It was found that cyanide significantly ($.005<P<.01$) stimulates the uptake of oxygen by giardia trophozoites.

The results with fluoride are more ambiguous but it should be noted that it took 10^{-2}M fluoride to actually kill giardia in growth experiments; lower concentrations only prevented growth.

Iodoacetate at 10^{-4}M concentration appeared to have little effect on giardia trophozoites, while 10^{-3}M caused complete inhibition.

Dinitrophenol at 10^{-5}M appeared to cause a small stimulation of oxygen uptake.

The small number of samples of fluoride, iodoacetate and dinitrophenol tested were insufficient to perform statistical analysis. The stimulation observed with 10^{-3}M fluoride may

have been an artifact of the particular experiment as one would not expect fluoride to stimulate oxygen consumption. The compound is known to react with a large number of metallo-enzymes (184) and certain cytrochromes (185) in the higher concentrations used here.

TABLE 7

Effects of Certain Metabolic Inhibitors on the Endogenous Oxygen Consumption of Giardia Trophozoites in Hanks' BSS: Oxygen Electrode Measurements

inhibitor	conc.	endogenous respiration	inhibitor respiration	percent inhibition
cyanide	10^{-5}M	14.5	17.2	+ 19
"	10^{-5}	14.5	22.7	+ 57
"	10^{-5}	1.8	8.3	+361
"	10^{-5}	4.0	6.0	+ 50
"	10^{-3}	15.0	16.0	+ 6
"	10^{-3}	17.5	22.5	+ 29
"	10^{-2}	7.5	9.0	+ 20
fluoride	10^{-4}	16.0	20.0	+ 25
"	10^{-3}	16.0	18.0	+ 13
"	10^{-2}	18.0	17.0	6
iodoacetate	10^{-4}	21.0	21.0	0
"	10^{-3}	8.0	0	100
"	10^{-3}	16.0	0	100
dinitrophenol	10-5	19.0	22.0	+ 16

Values for respiration are given as Δ/minute/10^8 organisms where Δ = change in instrument reading which indicates the rate of oxygen removed from the solution.

IV. WARBURG EXPERIMENTS

A. Endogenous oxygen consumption

All Warburg experiments included a control of protozoa with no added substrate; this constituted the control for endogenous respiration.

In table 8 are summarized the endogenous controls from several experiments to determine if some correlation could be made between the number of organisms present and the amount of oxygen consumed. The rate of oxygen consumption was calculated from the 30 minute reading and extrapolated to 60 minutes because the rate of oxygen uptake by trophozoites often decreases after 30 to 45 minutes (see fig.10). Therefore, the actual oxygen consumption will usually be less than the values calculated in table 8 . The endogenous 0_2 uptake data were examined to see if the values, obtained over a number of experiments and normalized to µl 0_2 uptake/hr/10^8 cells, were distributed normally. The values were found to be grouped in proportions similar to items in a normal curve, that is: 66.7% fell between ± 1 S.D. of the mean, 95.8% fell between ± 2 S.D. and 100% fell betweem ± 3 S.D. This is compared with a normal distribution where 68.27% of the items fall between ± 1 S.D., 95.45% fall between ± 2 S.D. and 99.73% fall between ± 3 S.D. of the mean.

TABLE 8

Endogenous Oxygen Consumption of Giardia Trophozoites in Hanks' BSS: Warburg Measurements.

cells/flask	O_2 uptake 30 min.	O_2 uptake µl/hr/10^8 cells
$8.4X10^7$	8	19
$7.0X10^7$	13	37
$7.8X10^7$	17	44
$7.8X10^7$	18	46
$4.3X10^7$	10	47
$7.8X10^7$	19	49
$7.8X10^7$	20	51
$3.2X10^7$	10	63
$7.5X10^7$	24	64
$5.1X10^7$	17	67
$2.5X10^7$	9	72
$9.4X10^7$	34	72
$8.7X10^7$	34	78
$1.2X10^8$	50	83
$6.8X10^7$	31	91
$1.2X10^8$	56	93
$5.7X10^7$	27	95
$1.1X10^8$	53	96
$1.1X10^8$	53	96
$8.7X10^7$	42	97
$4.5X10^7$	26	116
$7.0X10^7$	43	123
$2.9X10^7$	18	124
$7.2X10^7$	55	153
		mean 78

Values for O_2 uptake are expressed as µl O_2/flask/30 min. Calculations for µl O_2/hr/10^8 cells were based on 30 min. readings as described in section III A

Data from table 8 were used to construct a scatter plot comparing the number of organisms with the amount of oxygen consumed (see fig.8). In addition, a Pearson's correlation coefficient was derived to determine the extent of correlation. A value of 0.62 was obtained indicating only moderate correlation between number of organisms and amount of O_2 consumed. The low correlation emphasizes the importance of including endogenous controls in every experiment.

The observed variation from batch to batch of giardia prompted measuring the oxygen consumption of several dilutions of a single harvest of organisms. The results are shown in figure 9. In this case, as the number of organisms increased, oxygen consumption increased. A Pearson's correlation coefficient was derived for these data and was found to be 0.98. This very high correlation indicates that the amount of oxygen taken up was directly proportional to the number of organisms present.

B. Effect of glucose addition on oxygen uptake

In certain Warburg experiments, glucose was added to determine if it would stimulate giardia trophozoites to consume more oxygen; results of these experiments were presented in table 9 and figure 10. When glucose was added there was a consistant stimulation of about 20 to 25 percent over endogenous respiration whether 0.024M or 0.06M glucose was added.

Figure 8

Correlation of number of organisms with the amount of endogenous oxygen consumption: Warburg measurements.

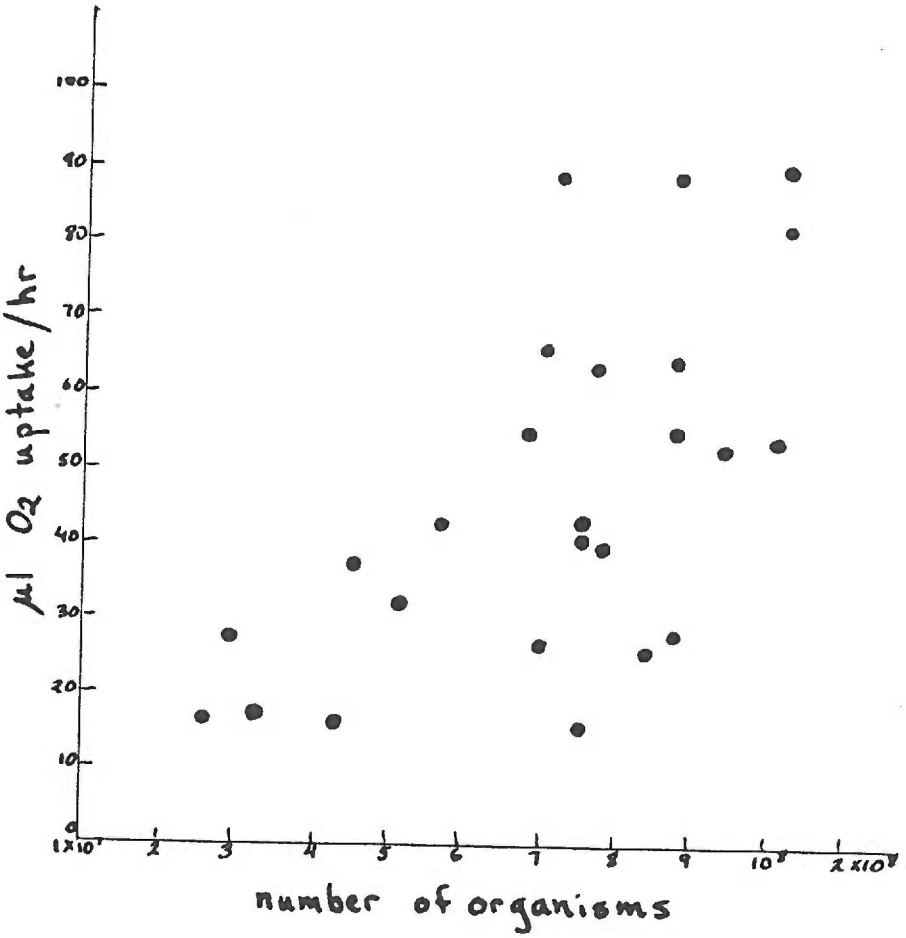

μl O2 uptake/hr
100
90
80
70
60
50
40
30
20
10
0
1X10⁷
2
3
4
5
6
7
8
9
10⁸
2 X10⁸
number of organisms

Figure 9

Endogenous oxygen consumption of three dilutions of the same harvest of Giardia trophozoites.

The following Warburg data were graphed:

number of organisms	$\mu l\ O_2$ uptake/hr
$8.30X10^6$	16
$1.66X10^7$	30
$2.49X10^7$	41

Each point is the average of two flasks. The correlation coefficient derived for these data was 0.98.

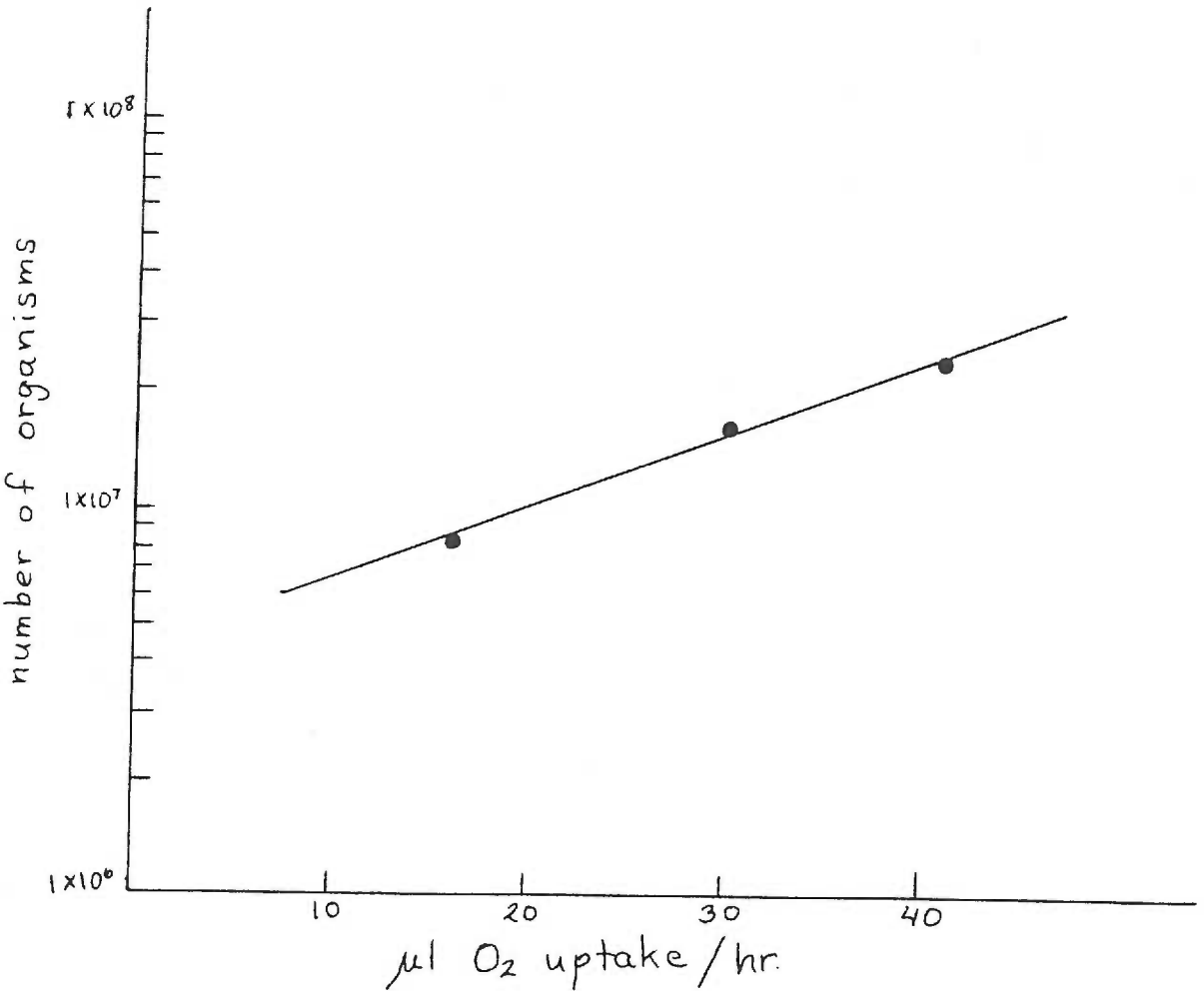

number of organisms
1X10⁸
1X10⁷
1X10⁶
10
20
30
40
µl O₂ uptake / hr.

TABLE 9

Effect of Glucose on the Oxygen Consumption of Giardia Trophozoites in Hanks' BSS: Warburg Measurement.

organisms flask	glucose conc.	endog. resp.	glucose resp.	percent stim.
$6.5X10^7$	0.06M	122	176	44
$8.4X10^7$	"	30	39	30
$1.2X10^7$	"	73	88	21
$1.2X10^8$	"	67	73	9
$8.4X10^7$	"	55	59	7
			Ave.=	26%
$3.2X10^7$	0.024M	53	63	19
$4.3X10^7$	"	37	49	32
$1.1X10^8$	"	48	55	15
$8.7X10^7$	"	62	75	21
$4.5X10^7$	"	82	98	20
			Ave.=	21%

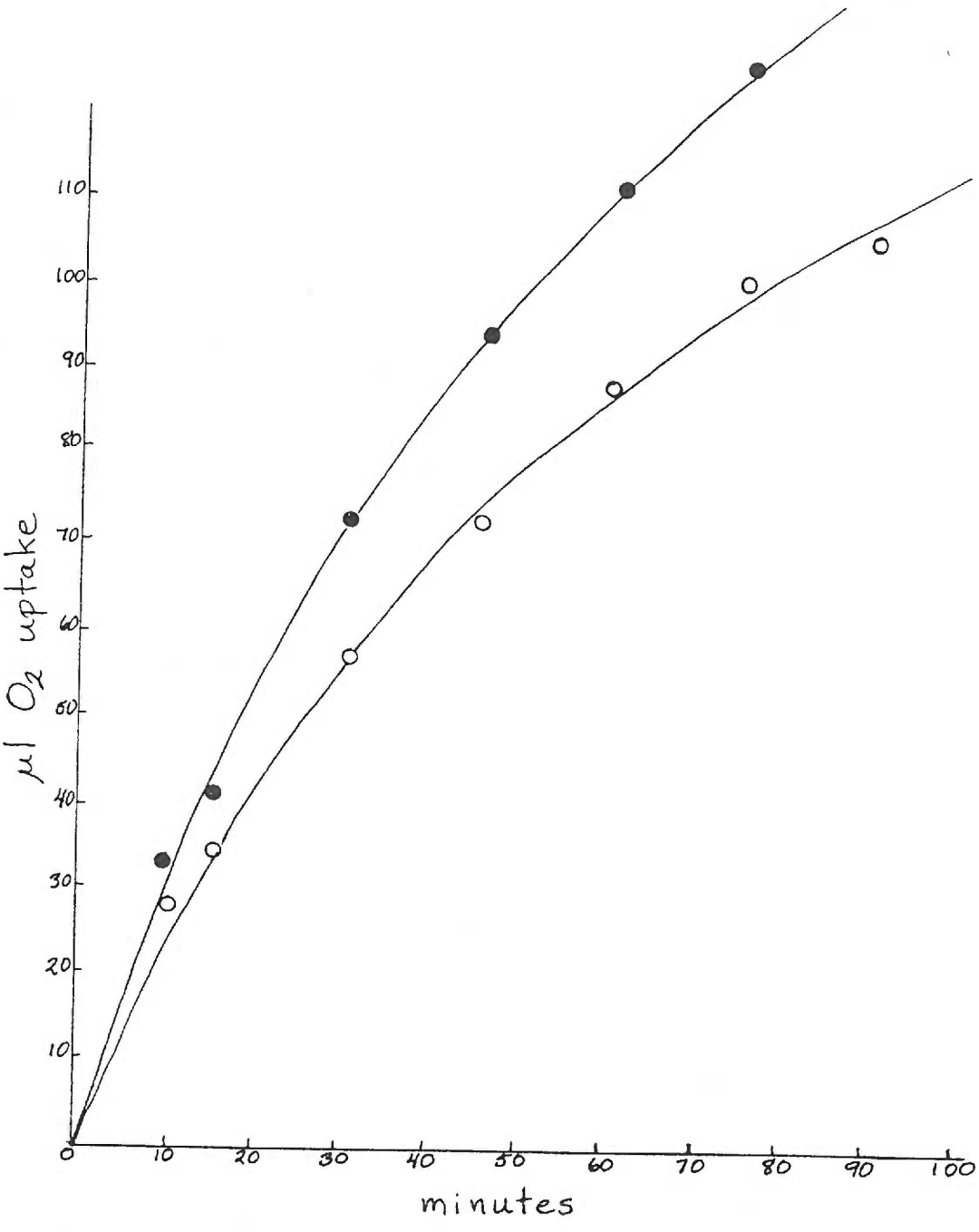
Values for respiration are given as µl O_2 uptake/hr/10^8 organisms. In paired t tests performed between endogenous and glucose respiration, t was significant at or below the 0.05 level.

Figure 10

A representative curve showing the effect of glucose on the oxygen consumption of giardia trophozoites in Hanks' BSS: Warburg measurement.

Open circles: Endogenous oxygen uptake

Closed circles: Oxygen uptake with 0.06M added glucose


μl O2 uptake
110
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
minutes

Separate paired t tests were preformed on the 0.024M and 0.06M data. The effect of the glucose was found to be signigicant at the .05 level.

C. Effect of cyanide on oxygen consumption

Warburg experiments were conducted to determine the effect of 10^{-4}M KCN on the ability of trophozoites to take up oxygen. Results are presented in table 10. Cyanide significantly stimulated the ability of giardia to consume oxygen above the endogenous level even when glucose was absent. Adding glucose did not always increase the stimulation. The phenomenon of cyanide-stimulated oxygen consumption has been observed in other parasites and will be discussed later.

D. Effect of fluoride on oxygen consumption

Warburg experiments were carried out to determine the effect of 10^{-2}M NaF on the ability of giardia to take up oxygen. The results (see table 11) indicate that NaF reduces oxygen uptake. Addition of glucose did not restore oxygen consumption to even endogenous levels.

TABLE 10

Effect of Cyanide on the Oxygen Consumption of Giardia Trophozoites in Hanks' BSS: Warburg Measurements.

organisms flask	endog. resp.	glucose resp.	KCN resp.	glucose + KCN	percent KCN stim.
$8.4X10^7$	41	47	77	61	83
$8.4X10^7$	36	46	73	44	102
$1.2X10^8$	59	72	78	74	32
$1.2X10^8$	54	75	59	79	9
$1.2X10^8$	61	69	55	78	-10
$1.2X10^8$	55	70	70	71	27
$9.4X10^7$	44	84	84	95	90
					Ave.= 48 %

Values given as µl O_2 uptake/hr/10^8 organisms. Each value is from a separate flask. Endogenous flasks contained no substrate (control), glucose flasks contained 0.06M glucose, KCN flasks contained 10^{-4}M KCN. In paired t tests between non-substrate (control) and substrate flasks, t was found to be significant at or below the .05 level in all cases. Percent KCN stimulation was calculated between endogenous respiration and KCN respiration.

TABLE 11

Effect of Fluoride on the Oxygen Consumption of Giardia Trophozoites in Hanks' BSS: Warburg Measurements

organisms flask	endog. resp.	glucose resp.	NaF resp.	glucose + NaF	percent inhib.
$8.7X10^7$	39	85	29	31	26
$8.7X10^7$	41	77	31	34	24
$4.5X10^7$	44	68	31	39	12
$4.5X10^7$	44	59	33	29	34
				Ave.=	24 %

Values given as µl O_2 uptake/hr/10^8 organisms. Each value is from a separate flask. Endogenous flasks contained no substrate (control), glucose flasks contained 0.06M glucose, NaF flasks contained 10^{-2}M fluoride and glucose + NaF flasks contained 0.06M glucose plus 10^{-2}M fluoride. In paired t tests between non-substrate (control) and substrate flasks, t was found to be significant at or below the .05 level in all cases. Percent NaF inhibition was calculated between endogenous respiration and NaF respiration.

E. Prolonging oxygen consumption in the Warburg flask

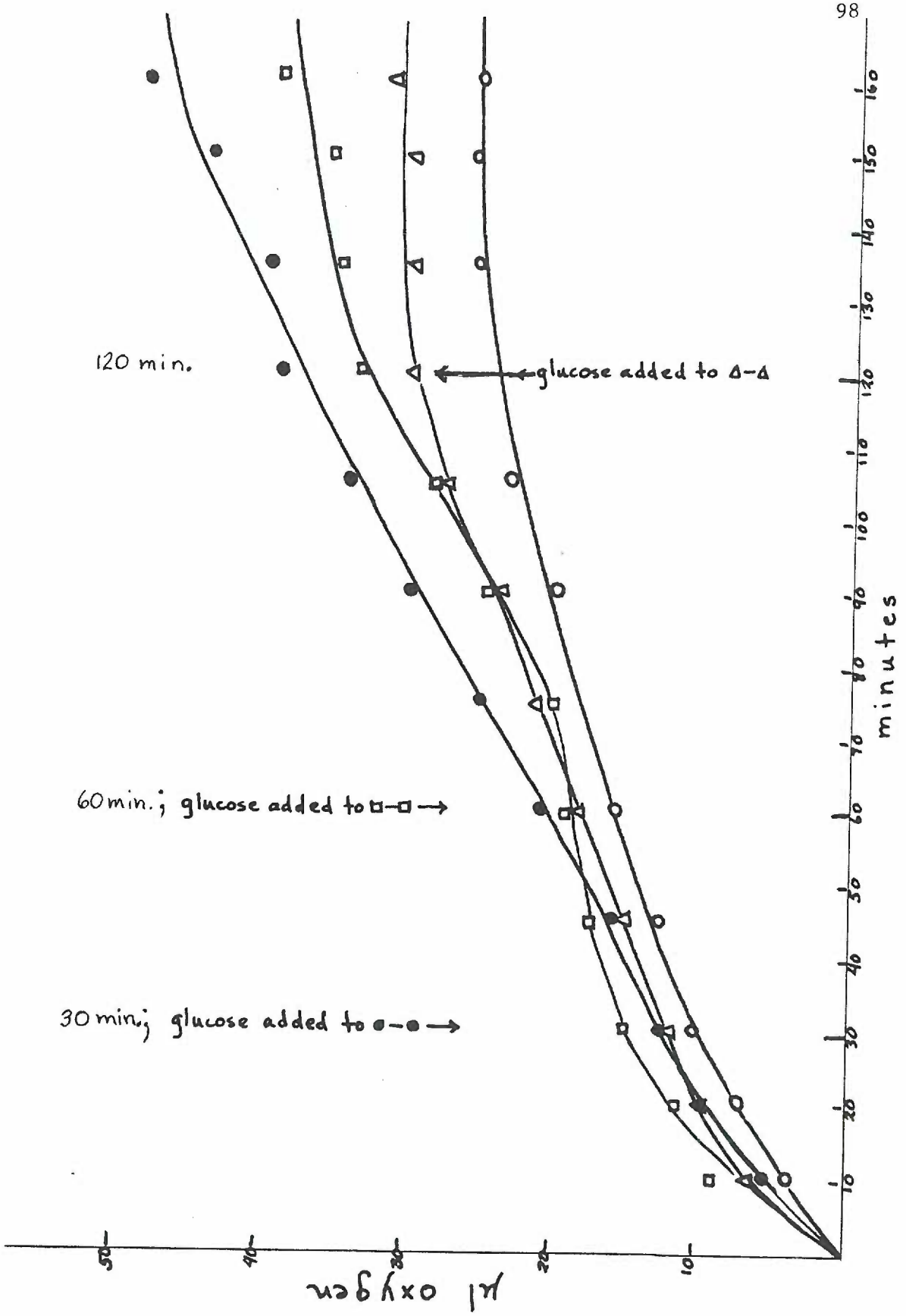
It has been noted that oxygen consumption by the trophozoites begins to decline after about 30 minutes in the Warburg flask. The following experiment was designed to determine if glucose added at various times to giardia in a Warburg flask would restore their ability to take up oxygen. The results, plotted in Figure 11, show that adding glucose at 30 and 60 minutes did restore oxygen consumption. It allowed the organisms to take up more oxygen than the "endogenous" organisms in the same time period. Adding glucose at 120 minutes, however, failed to stimulate the organisms to take up much more oxygen.

Figure 11

Effect of adding glucose (0.024M) to trophozoites in Hanks' BSS at various times in the Warburg flask.

Glucose was added to the organisms at 30, 60, and 120 minutes. None of the samples contained glucose at time 0.

Open circles: Endogenous oxygen consumption
Close circles: Glucose added at 30 minutes
Squares: Glucose added at 60 minutes
Triangles: Glucose added at 120 minutes
$4.5X10^7$ organisms/flask


120 min.
glucose added to Δ–Δ
60 min.; glucose added to □–□ →
30 min.; glucose added to ●–● →
minutes
μl oxygen

V. GLUCOSE CONSUMPTION EXPERIMENTS

Rate of glucose uptake by giardia trophozoites in Hanks' solution

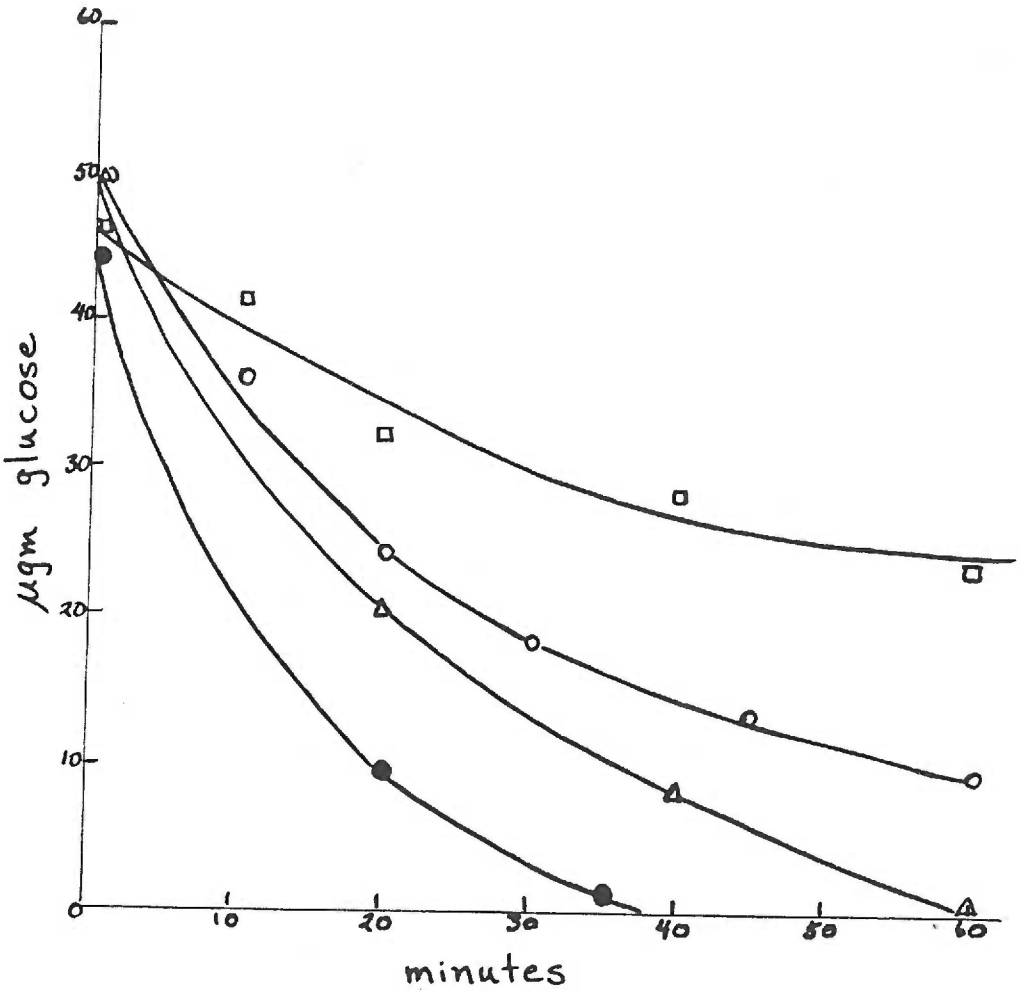
Experiments were set up in which 50 µgm/ml of glucose was added to samples of giardia trophozoites in Hanks' solution. The samples were incubated at 37 C. At various incubation times, ranging from 0 to 60 minutes, samples were tested for glucose remaining in solution by the glucose oxidase method. Controls consisted of samples with no organisms (the standard) and samples with killed organisms (to determine any physical glucose absorption). The difference between these two controls was subtracted from each test sample. The results of four experiments are presented in Figure 12. The rate of glucose consumption ranged from 152 to 455 µgm/hr/10^8 organisms. There was found to be low correlation between number of organisms and amount of glucose consumed (r=0.24). Serially diluted samples of the same harvest might have given linearity of glucose uptake/# organisms as with 0_2 uptake (see Fig.9).

Figure 12

Glucose consumption by Giardia trophozoites in Hanks' BSS: Glucose oxidase measurement.

Samples were given 50 µgm/ml of glucose. The amount of glucose remaining in solution at various times was measured.

Open circles: $2.25X10^7$ organisms/ml
Closed circles: $1.45X10^7$ organisms/ml
Squares: $1.45X10^7$ organisms/ml
Triangles: $2.2X10^7$ organisms/ml


60
50
40
30
20
10
0
μgm glucose
10
20
30
40
50
60
minutes

VI. PROTEIN ASSAYS

To determine the protein content of giardia trophozoites, small samples of trophozoites from different experiments were removed and frozen before any chemicals had been added. When a number of such samples had been collected, their protein content was determined.

A. Comparison of Lowry and Nesslerization methods

A nesslerization technique was used to check the accuracy of the Lowry method of determining protein in giardia. It is known that the Lowry method may give different amounts of color for different proteins. Table 2 compares the two methods and it can be seen that they agree very well. The Lowry method, the easier and faster of the two, was used for all subsequent protein determinations. The method is sensitive to 5 µgm protein.

B. Correlation of number of organisms and protein content

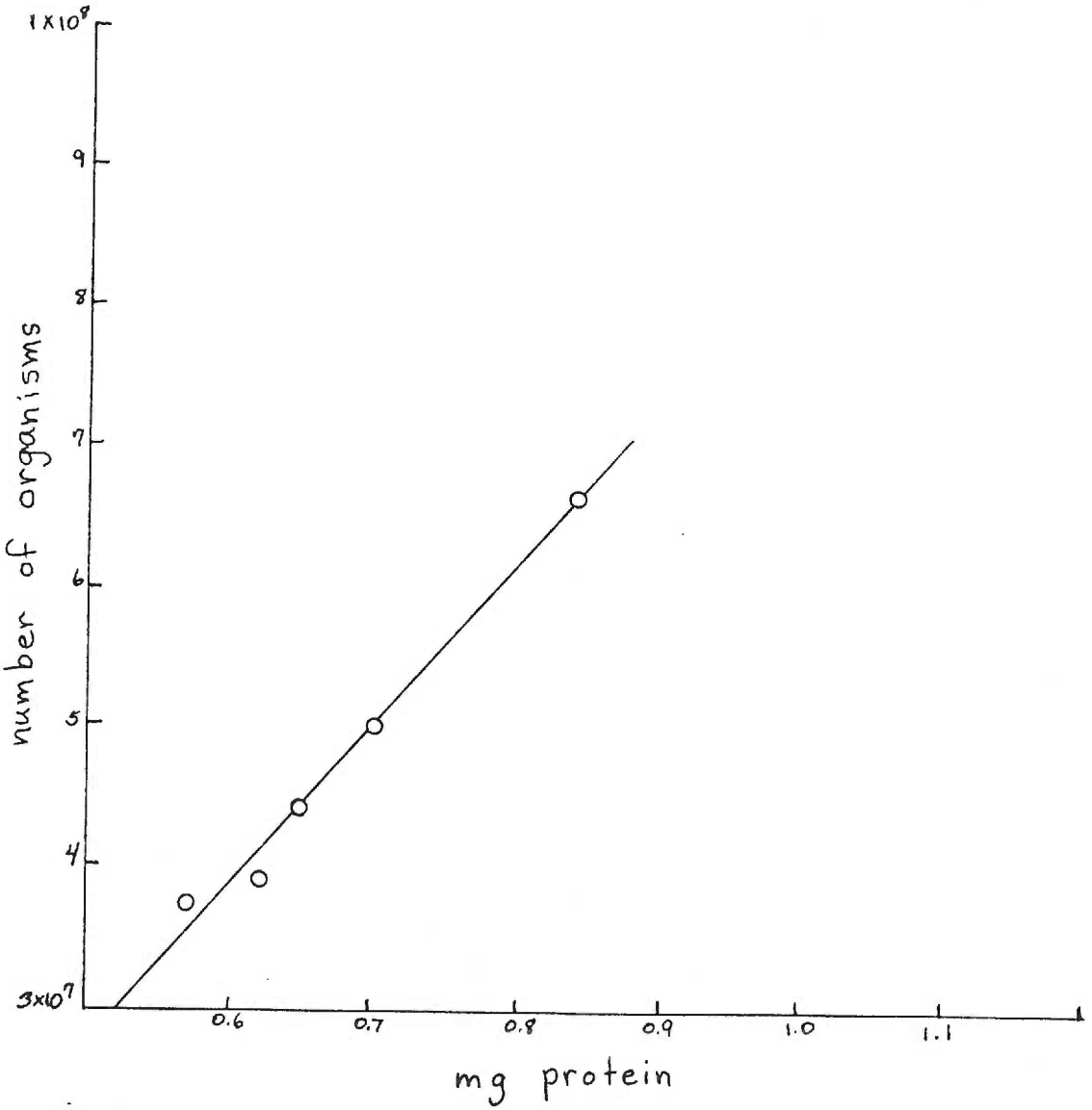
An attempt was made to correlate the actual haemocytometer counts of organisms with the protein determinations of the same samples. These data are presented in Figure 13. It can be seen that, in most cases, the amount of protein was directly proportional to the number of cells present; deviations from

this proportionality will be discussed later.

The average protein content of 10^8 giardia trophozoites was calculated as 1.46 mg with a standard deviation of 0.11 mg.

Figure 13

Correlation of number of organisms with protein content of giardia trophozoites in Hanks' BSS: Lowry protein determinations.


1X10^8
9
8
7
6
5
4
3X10^7
number of organisms
0.6
0.7
0.8
0.9
1.0
1.1
mg protein

VII. POLYSACCHARIDE

It was thought possible that the high endogenous respiration observed in giardia (see table 6 and table 8) might be due to the oxidative breakdown of glycogen reserves. Several electron microscope studies of Giardia report the presence of glycogen granules in the cytoplasm (12,162). Therefore, experiments were run to determine the possible presence and behavior of a polysaccharide pool.

A. Determination of polysaccharide content of giardia trophozoites

Several samples of trophozoites were assayed for glycogen. The results are shown in table 12. It is apparent that under the cultural conditions used here, giardia trophozoites do contain polysaccharide.

B. Rate of intracellular glycogen consumption by giardia trophozoites

To determine if giardia consumed their intracellular reserves of glycogen while suspended in Hanks' BSS, samples of organisms in Hanks' were incubated at 37 C without glucose; at various times samples were removed and assayed for glycogen. Results of two of these experiments are recorded in table 13.

TABLE 12

Glycogen Content of Giardia Trophozoites

no. of organisms	age of organisms	µgm of glycogen	glycogen µgm/10^8 organisms
$2.0X10^7$	72 hr.	21	105
$5.2X10^7$	72 hr.	17	33
$4.1X10^7$	96 hr.	33	81
$3.8X10^7$	96 hr.	460	1210

TABLE 13

Rate of Endogenous Glycogen Utilization by Giardia Trophozoites in Hanks' BSS

no. of organisms	incubation time (min) 0'	15'	30'	µgm glycogen/hr/ 10^8 organisms
$4.1X10^7$	33	33	26	67
$3.8X10^7$	460	459	360	53

Values under "incubation time" are µgm glycogen remaining in the cells.

VIII. RADIO-LACTATE EXPERIMENTS

A. Lactate and CO_2 production from uniformly labeled (UL) ^{14}C-glucose

Experiments were set up in which the production of lactic acid and CO_2 from uniformly labeled ^{14}C-glucose was monitored. Table 14 shows the results. The values for the "background" (no cells) and "dead cell" samples were subtracted from the "live cell" sample before calculations were performed to determine the amount of lactate and CO_2 produced. The ratio of micromoles of lactate to micromoles of CO_2 produced was calculated. This averaged about 1.14 μM of lactate per μM CO_2.

B. Effects of metabolic inhibitors on production of lactate and CO_2

Experiments were run in which the effects of certain metabolic inhibitors on the production of CO_2 and lactate from UL-^{14}C-glucose were studied. The results are presented in Tables 15 through 18. Fluoride inhibited lactate and CO_2 production at 10^{-1} and 10^{-2}M concentration. Carbon dioxide but not lactate was inhibited at 10^{-3}M fluoride. Iodoacetate inhibited lactate and CO_2 production almost totally at 0.07, 0.007, and 0.0007M concentrations. Cyanide inhibited lactate

at 10^{-1}M and 10^{-2}M but inhibited CO_2 only at 10^{-1}M KCN. The stimulating effect of cyanide on lactate and CO_2 production at 10^{-3}M concentration was not significant at the .05 level according to a t test. However, a larger number of experiments is needed to accurately determine the effect of cyanide at this concentration due to wide variation between samples.

Lactate production was inhibited by 10^{-4}, 10^{-5}, and 10^{-6}M 8-OH-Quinalin while the formation of CO_2 was not appreciably affected.

C. Lactate and CO_2 production from ^{14}C-1-glucose and ^{14}C-6-glucose

A very preliminary experiment was conducted using ^{14}C-1-glucose and ^{14}C-6-glucose instead of ^{14}C-UL glucose. The effect of fluoride on CO_2 and lactate production was also studied. Although the number of samples was very small, and therefore the conclusions rather speculative, the results were interesting. Lactate and CO_2 were produced from both the C-1 and C-6 position (Tables 19 and 20). Lactate was produced nearly equally from the C-1 and C-6 position. However, more CO_2 was produced from the C-1 position than the C-6. Fluoride inhibited lactate and CO_2 production differently, being a more effective lactate than CO_2 inhibitor.

TABLE 14

Production of Lactic Acid and CO_2 from Uniformly Labeled (UL) ^{14}C-Glucose by Giardia Trophozoites in Hanks' BSS: Radio-lactate Method

organisms sample	CO_2 CPM	$\frac{\mu M\ CO_2}{10^8 \text{ organisms}}$	lac CPM	$\frac{\mu M \text{ lac}}{10^8 \text{ organisms}}$	$\frac{\mu M \text{ lac}}{\mu M\ CO_2}$
$4.3X10^7$	249483	.014	733000	.013	.93
$4.3X10^7$	271858	.015	850700	.015	1.00
$4.2X10^7$	256608	.014	823500	.015	1.07
$2.3X10^7$	149927	.008	524100	.009	1.13
$2.3X10^7$	114240	.006	535600	.010	1.67
$1.4X10^7$	138784	.008	404100	.007	.88
$1.4X10^7$	117670	.006	292100	.005	.93
$1.2X10^7$	139449	.008	467400	.008	1.00
$1.2X10^7$	153456	.008	483400	.009	1.13
$1.7X10^7$	118678	.006	478700	.009	1.50
$5.0X10^6$	124902	.007	567000	.010	1.43
				Ave.=	1.14

CPM= counts per minute
lac= lactate

Samples contained 0.5 ml of organisms in Hanks' BSS, 0.4 ml of Hanks' BSS and 2.7 µgm UL-glucose. At the end of 30 minutes incubation, 0.1 ml of 25% TCA was added to kill the protozoa and release CO_2. Controls consisted of samples with no organisms and samples with dead organisms. There was high correlation between the µM of CO_2 and the µM of lactate produced (r=0.91).

TABLE 15

Effect of Fluoride on the Production of Lactate and CO_2 from UL-^{14}C-Glucose by Giardia Trophozoites in Hanks' BSS: Radiolactate Method

Control		10^{-1}MF$^-$		10^{-2}MF$^-$		10^{-3}MF	
lac	CO_2	lac	CO_2	lac	CO_2	lac	CO_2
.47	.46	.04	.03	.20	.15	.33	.37
.58	.47	---	---	.35	.22	.49	.34
.04	.02	.001	.002	.02	.01	.03	.02
.04	.02	.0001	.003	.01	.01	---	---
3.82	3.56	.05	.200	1.02	.85	---	---
3.90	2.70	.02	.06	1.43	1.07	3.89	2.66
4.82	5.41	.01	.09	1.81	2.57	3.99	4.95
3.49	4.60	.01	.46	1.92	1.56	2.54	4.48
6.52	6.37	.10	.26	2.64	2.78	6.11	6.96
6.73	7.00	.04	.19	2.38	2.50	6.03	6.82
4.70	3.82	.11	.36	1.92	1.74	4.84	3.92
4.98	2.89	.17	.37	2.17	1.64	4.28	4.49
3.61	1.82	.05	.51	2.03	1.32	3.68	2.99
3.13	3.34	.01	.42	2.19	1.59	3.01	2.08
$\bar{x}$ 3.35	3.03	.05	.23	1.44	1.27	3.18	2.89
% inhib		99	93	57	58	5	5

Values in the table are given as $\mu MX10^{-2}$ lactate or $CO_2/10^8$ organisms. T tests performed on the data showed that at all concentrations of fluoride, lactate and CO_2 values were significantly different from the control values except lactate values at $10^{-3}MF^-$. Values were considered significant at or below the 0.05 level.

TABLE 16

Effect of Iodoacetate on the Production of Lactate and CO_2 from UL-^{14}C-Glucose by Giardia Trophozoites in Hanks' BSS: Radio-lactate Method.

Control		.07M Iodo.		.007M Iodo.		.0007M Iodo.	
lac	CO_2	lac	CO_2	lac	CO_2	lac	CO_2
3.82	3.56	0	0	0	.09	.008	.37
3.90	2.70	0	0	0	.08	.017	1.17
4.82	5.41	0	0	0	.03	.012	1.31
3.49	4.60	0	0	0	.01	.010	1.03
6.52	6.37	0	.08	0	.09	.006	.68
6.73	7.00	0	0	0	.09	.003	.59
4.70	3.82	0	.05	0	.12	.003	.46
4.98	2.89	0	.05	0	.11	.003	.85
$\bar{x}$ 4.87	4.54	0	.02	0	.08	.008	.81
% inhibition		100	99.6	100	98	99.8	82

Values in table are $\mu MX10^{-2}$ lactate or $CO_2/10^8$ organisms. T tests performed on the above data showed that all lactate and CO_2 values were significantly different from control values. Values were considered significant at or below the 0.05 level.

TABLE 17

Effect of Cyanide on the Production of Lactate and CO_2 from UL ^{14}-C-Glucose by Giardia Trophozoites in Hanks' BSS: Radiolactate Method.

Control		10^{-1}M Cyanide		10^{-2}M Cyanide		10^{-3}M Cyanide	
lac	CO_2	lac	CO_2	lac	CO_2	lac	CO_2
3.82	3.56	0	.003	.22	2.97	4.84	---
3.90	2.70	.037	.003	.27	2.97	4.36	3.58
4.82	5.41	.001	.109	.44	.62	6.53	6.24
3.49	4.60	0	.011	.41	1.09	7.59	8.03
$\bar{x}$ 4.01	4.07	.009	.032	.34	1.91	5.83	5.95
% inhib.		99.8	99.2	91.5	53	+45	+46

Values in the table are $\mu MX10^{-2}$ lactate or $CO_2/10^8$ organisms. T tests performed on the above data showed that lactate values were significantly different from control values at 10^{-1}M and 10^{-2}M CN^- but not at 10^{-3}M CN. CO_2 values were significantly different at 10^{-1}M CN but not at 10^{-2}M and 10^{-3}M CN. Values were considered significant at or below the 0.05 level.

TABLE 18

Effect of 8-OH-Quinolin on the Production of Lactate and CO_2 from UL-^{14}C-Glucose by Giardia Trophozoites in Hanks' BSS: Radio-lactate Method.

Control		10^{-4}M 8-OH-Quin.		10^{-5}M 8-OH-Quin.		10^{-6}M 8-OH-Quin.	
lac	CO_2	lac	CO_2	lac	CO_2	lac	CO_2
6.50	6.32	3.84	3.09	3.48	5.87	4.71	6.28
6.73	7.00	5.13	5.41	3.41	5.82	5.04	6.13
4.71	3.82	3.98	5.23	4.18	5.33	5.68	5.23
4.98	2.89	3.74	5.10	3.96	5.62	3.34	4.59
$\bar{x}$ 5.73	5.01	4.17	4.71	3.76	5.66	4.19	5.56
% inhib.		27	6	34	+13	27	+11

Values in table are $\mu MX10^{-2}$ lactate or $CO_2/10^8$ organisms. T tests on the above data showed that lactate production at all concentrations of 8-OH-Quinolin was significantly different from control values. CO_2 production was not significantly different from controls at any concentrations of 8-OH-Quinolin. Values were considered significant at or below the .05 level.

TABLE 19

Effect of Fluoride on Production of Lactate and CO_2 from ^{14}-C-1-Glucose by Giardia Trophozoites in Hanks' BSS: Radio-lactate Method.

Control		$10^{-2}MF^-$		$10^{-3}MF^-$		$10^{-4}MF^-$	
lac	CO_2	lac	CO_2	lac	CO_2	lac	CO_2
2.36	2.14	1.11	1.71	1.93	2.41	2.07	2.36
2.35	2.02	1.03	1.74	2.07	2.58	2.14	2.02
$\bar{x}$ 2.36	2.08	1.07	1.73	2.00	2.50	2.11	2.19
% inhib.		55	17	15	+20	11	+5

Values in the table are $\mu MX10^{-2}$ lactate or $CO_2/10^8$ organisms. T tests on the above data showed that all lactate values were significantly different from control values. CO_2 values were significantly only at $10^{-2}MF^-$.

TABLE 20

Effect of Fluoride on Production of Lactate and CO_2 from ^{14}-C-6-Glucose by Giardia Trophozoites in Hanks' BSS: Radio-lactate Method.

Control		$10^{-2}MF^-$		$10^{-3}MF^-$		$10^{-4}MF^-$	
lac	CO_2	lac	CO_2	lac	CO_2	lac	CO_2
2.57	1.59	1.14	1.15	2.09	1.33	2.33	1.36
2.78	1.57	1.23	1.18	2.32	1.58	2.57	1.49
$\bar{x}$ 2.68	1.58	1.19	1.17	2.21	1.46	2.45	1.43
% inhib.		56	26	17	8	9	9

Values in table are $\mu MX10^{-3}$ lactate or $CO_2/10^8$ organisms. T tests on the above data showed that lactate values were significantly different from controls at all concentrations of F^-. CO_2 values were significant only at $10^{-2}MF^-$. Values were considered significant at or below the .05 level.

D. Distribution of radioactive material in radio-lactate experiments

Analysis of the data from the radio-lactate experiments showed that not all of the label (counts per minute, or CPM) given the cells was recovered as CO_2 or lactate (see Table 21). Therefore, an experiment was set up to determine the distribution of label in the sample at the end of 30 minutes incubation. The protocol followed was the same as for all radio-lactate experiments except that at the end of the incubation period the cells were spun down and 0.1 ml of the supernatant counted with no further treatment. The cells were washed three times with Hanks' by centrifugation, the pellet resuspended in 0.2 ml of Hanks', and 0.1 ml pipetted onto a piece of Whatman #1 paper. This was allowed to dry, then counted. The CO_2 and lactate productions were determined in duplicate samples in the usual way. Table 22 shows the distribution of the label (CMP) in the sample.

TABLE 21

RECOVERY OF RADIOACTIVE MATERIAL FROM RADIO-LACTATE EXPERIMENTS

CPM added	CPM recovered	CPM missing	percent recovery
$1.76X10^6$	$1.2\ X10^6$	$.56X10^6$	68
$3.5\ X10^5$	$2.6\ X10^5$	$.9\ X10^5$	74
$1.76X10^6$	$.72X10^6$	$1.04X10^6$	41
$1.76X10^6$	$.51X10^6$	$1.25X10^6$	29
$1.76X10^6$	$.65X10^6$	$1.1\ X10^6$	37
$1.76X10^6$	$1.3\ X10^6$	$.46X10^6$	74

CPM: Counts per minute. "CPM added" was the amount of label added as UL-glucose. "CPM recovered" was the amount of label measures as CO_2 and lactate. "CPM missing" was the amount of label that could not be accounted for as CO_2 and lactate. Number of cells used in these experiments ranged from $1.2X10^7$ to $4.3X10^7$ cells/ml.

TABLE 22

DISTRIBUTION OF RADIOACTIVE MATERIAL IN RADIO-LACTATE EXPERIMENTS

fraction of sample	CPM	% of total
CPM ADDED	$1.76X10^6$	100.0
cell bound	$.01X10^6$	0.6
CO_2	$.20X10^6$	11.4
lactate	$.70X10^6$	39.8
supernatant (-lactate CPM)	$.60X10^6$	34.1
washings	$.24X10^6$	14.2
missing	$.01X10^6$	.6
	Total	100.7

CPM "added" was the amount of label added as UL-glucose; "cell bound" CPM was the amount of label that could not be removed from organisms by washing. Lactate and CO_2 label were determined by the radio-lactate method. "Supernatant" CPM was the amount of label present in the incubation fluid at the end of 30 minutes minus the CPM due to lactate.

IX. CHROMATOGRAPHY AND AUTORADIOGRAPHY

A. Chromatography of radio-lactate supernatants

Samples of the supernatants from the radio-lactate experiments were taken before the copper-calcium treatment, and chromatographed as described in the Methods section. Figure 14 is a tracing of a typical radiogram obtained from a sample of cells exposed to UL-glucose for 30 minutes. The spots are numbered for identification in Table 23, which shows the distribution of the label in the entire sample, including CO_2 and lactate.

Some samples were taken after the copper-calcium treatment and chromatographed. These chromatograms typically showed only one spot in the position where lactate should be.

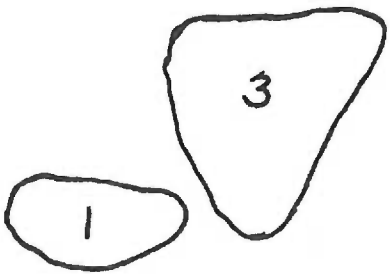
Note: Spot number 5 was not included in the table because it was later identified as lactate, which could be accounted for by the radio-lactate measurement.

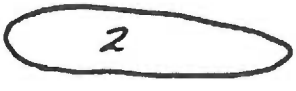
Figure 14

Tracing of a representative autoradiogram of the chromatographed supernatant fluid from a sample of *Giardia* trophozoites exposed to UL-glucose in Hanks' BSS for 30 minutes.


5


4


3
1


2


0

TABLE 23

DISTRIBUTION OF RADIOACTIVE MATERIAL IN SAMPLES OF GIARDIA TROPHOZOITES EXPOSED TO UL GLUCOSE IN HANKS' BSS

radioactive material	% of total counts
UL glucose added	100.0
CO_2	9.9 ± 1
lactate	30.6 ± 1
chromatography spot	
0 (origin)	2.8 ± 1
1	32.9 ± 15
2	5.9 ± 1
3	13.1 ± 5
4	0.8 ± 0.1
cellular retention	0.7 ± 0.1
unaccounted	9.0 ± 5
Total	105.7

Samples contained 2.8 - $4.1X10^7$ organisms/ml and 1.24 to $1.5X10^6$ CPM of UL glucose. Lactate and CO_2 were determined by the radio-lactate method, chromatographed substances (spots) were determined by autoradiography and scintillation counting, and intracellular retention of label was measured as described in section VII E.

Note: Spot number 5 was not included in table because it was identified as lactate, which could be accounted for by radio-lactate measurement.

B. Identification of chromatographic spots

Spot number 1 was found to be glucose; spot number 5 was lactate. These were determined by comparing the autoradiograms with standard compounds chromatographed in the same manner as the radioactive samples. Co-chromatography of the radioactive samples and the suspected compounds confirmed the identity of the spots.

Spot number 2 was found to run in the same manner as such phosphorylated compounds as glucose-6-phosphate, fructose-6-phosphate and ribose-5-phosphate. Further identification was not attempted.

Direct spraying of the chromatograms with ninhydrin identified spots 3 and 4 as amines. When larger amounts of supernatant were chromatographed, as many as 13 to 14 amino acid spots of various intensities could be found. Time did not allow complete identification of any amino acid spot.

X. NADH OXIDIZING SYSTEM

Wellerson (36) reported that the oxygen consumption of T. vaginalis could be accounted for by the organism's ability to oxidize NADH. To determine if this could also be the case with G. duodenalis, an extract of trophozoites was assayed for its ability to oxidize NADH. It was found that giardia do indeed oxidize NADH. The specific activity of the extract assayed was 0.26 µmoles NADH/min/µgm protein. It was found that 3.2×10^6 organisms utilized 0.0116 µmoles NADH, which is equivalent to 0.26 µmoles O_2. On the basis of this, it was calculated that 1×10^8 organisms would utilize 484 µl O_2/hr. This is about twice the rate of the highest oxygen consumption measured with the oxygen electrode.

XI. ATP ASSAY

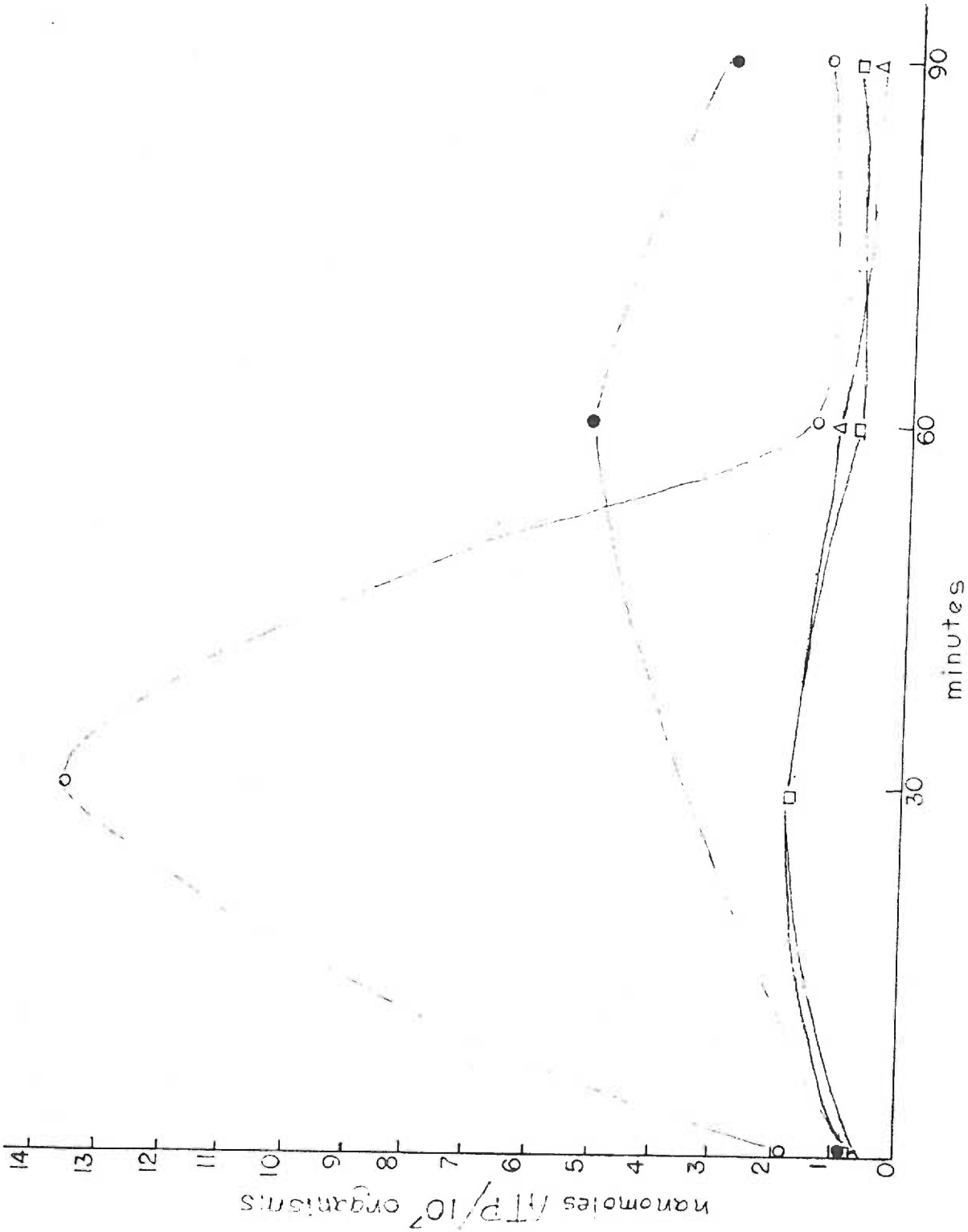
The results of ATP assays on a series of trophozoite samples which were either aerobic or anaerobic and to which glucose had been added or deleted are plotted in Figure 15.

Addition of glucose in the presence or absence of air increased the amount of ATP in the organisms. More ATP was produced in an aerobic environment.

Figure 15

Amount of ATP produced by Giardia trophozoites after 30, 60 and 90 minutes of incubation in Hanks' BSS.

Open circles: Glucose present (1 mg/ml); air present
Closed circles: Glucose present (1 mg/ml); air absent
Triangles: Glucose absent; air present
Squares: Glucose absent; air absent


nanomoles ΔTP/10⁷ organisms
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
30
60
90
minutes

XII. CYTOCHROME ASSAY

Figure 16 is a tracing of the difference spectrum obtained from a suspension of disrupted giardia trophozoites. The tracing was made from the spectrum obtained immediately after dithionate was added to the sample. It can be seen that the suspension absorbed fairly strongly in the regions where cytochromes c (about 520 mμ), b (560 mμ) and a (585-590 mμ) would be (186).

Figure 16

Difference spectrum (450-650 mμ) of an extract of Giardia trophozoites. ($1.3X10^8$ cells/ml)

One cuvette contained untreated extract, the other contained the extract plus a few crystals of sodium dithionate. The top spectrum was made immediately after reduction of one sample; the bottom spectrum was taken 5 minutes later. These are exact tracings from the originally recorded spectra.

Note: Instrument set on highest gain level due to low concentration of measured material.

optical density
.10
.09
.08
.04
.03
.02
450
500
550
600
650
wavelength (mμ)
~412
~519
~558
~587

DISCUSSION

Enviornmental factors affecting the growth and metabolism of giardia

At this point it might be well to look once again at the way of life of giardia before considering the biochemistry and physiology of the organism. First, the organism is a parasite; it relies on its host for food, protection and transportation (dissemination). Second, it lives in an environment low in oxygen tension (103). Third, it is presumbed that, in the distant past, its precursors were free-living (171). It is likely that giardia evolved from an aerobic organism. The parasite may have retained many of the metabolic systems of its ancestors. Over millions of years as giardia became better adapted to its host, it may have developed, through natural selection, metabolic systems that were advantageous to living in the gut. That is, enzyme systems that allow it to grow anaerobically or in a low oxygen tension. Depending upon how long giardia have been adapted to a parasitic life-style, it may or may not have lost its ability to utilize oxygen in a conventional manner.

It should also be remembered that giardia form cysts which are shed by the host into the external environment. This environment can be high in oxygen. The cyst is a resistant form of the parasite but nonetheless it is active metabolically. Division takes place inside the cyst, an energy requiring step. It could be possible that aerobic metabolism is necessary for the

survival of the cyst in the external environment. This is speculation as nothing is known of the metabloism of giardia cysts. It is known however that in some parasitic protozoa the metabolism in the cyst stage may be profoundly different from that in the trophozoite stage (125).

From the present study G. duodenalis appeares to be a facultative organism capable of both aerobic and anaerobic metabolism with leanings toward a fermentative metabolism. Although oxygen is consumed and CO_2 is produced under aerobic conditions, acid is also produced. The organism must be cultured in an essentially anaerobic environment. Freshly autoclaved medium must be used before much oxygen has diffused into it and the culture vessel must have as much air excluded as possible. If a large air space is left in the culture vessel the organism fail to multiply even in the presence of a reducing agent.

There may be several explanations for this apparent paradox. Oxygen may poison an enzyme or enzyme system necessary for giardial growth and multiplication. An example of such a mechanism is offered by Davis et. al (172).

> For example, succinate is required for the biosynthesis of several animo acids, and in faculative organisms this compound is synthesized under aerobic conditions by the oxidation of α-ketoglutarate in the tricarboxylate cycle; but anaerobically succinate is made by reduction of fumarate, through reversal of part of the cycle...In an E. coli mutant that has lost α-ketoglutarate oxidase anaerobic growth is still normal, but the addition of oxygen immediately stops growth unless succinate or its

biosynthetic products are provided. Oxygen thus "poisons" the flavoprotein fumarate reductase, presumably by keeping it too completely oxidized.

Giardia may have normal oxidative metabolism but lack catalase which breaks down peroxides formed through a conventional electron transport system. In trichomonads oxygen uptake is probably mediated by flavoproteins. T. foetus has catalase and therefore is insensitive to the toxic effect of peroxides. T. vaginalis and T. gallinae, however, depend on ascorbic acid or the catalase of serum in the medium to detoxify peroxides when grown under aerobic conditions (113).

Another possibility is that giardia lack superoxide dismutase (SOD). This enzyme apparently plays an important roll in protecting cells against the deleterious effects of the free radical O_2^- (superoxide radical). This radical is thought to be mutagenic. SOD converts the superoxide radical to peroxide by:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The enzyme is thought to be ubiquitous in aerobic cells and, like catalase, absent only in obligate anaerobes (160). SOD has been found in trichomonads and is at least partly associated with the organisms' redox organelle, the hydrogenosome. Many of the enzymes of the hydrogenosome are very sensitive to oxygen. It is thought that this explains the presence of SOD in the particles (122). Recent work by Lindmark indicates that

giardia do have SOD (188).

More insight into this problem was given by Malcom Smith in a discussion of the oxygen requirements of intestinal parasites (103). Many intestinal parasites that had long been thought to have strictly anaerobic metabolism, in actuality contained the full complement of TCA enzymes and were capable of oxidative metabolism. According to Smith, preconceived ideas about the anaerobiosis of the gut led workers to assume that oxidative metabolism was merely an evolutionary artifact, or that the enzymes had different functions under anaerobic conditions. The fact is that the gut is not completely anaerobic; measurements quoted by Smith show that oxygen may be present in concentrations as high as one quarter to one third that of air (40 to 50 mm partial pressure). The concentration of oxygen appears to exist as a gradient in the intestine; it is highest near the villi and close to zero in the center of the lumen. This would probably afford organisms such as giardia which attach to the villi, a very good source of oxygen. At the same time, the redox potential may be kept fairly low by the action of bacteria. Thus the oxygen sensitivity of the organism in culture may be a reflection of the necessity for a low redox potential and a lower oxygen concentration that that of air.

Other possible explanations of the sensitivity of giardia to oxygen may be related to the redox potential of the medium. Even when oxygen is excluded from culture vessels, a reducing

agent is necessary in the medium to allow giardial growth. The importance of the proper redox potential for the culture of cells and organisms has been known for some time (100,101). As noted by Jahn:

> It is well known that the optimal oxidation-reduction potential of anaerobic bacteria is quite different from that of aerobic bacteria, and recent work has shown that this difference is not due entirely to absence of oxygen. Oxygen-free media may be poised at a level which is too high to allow the growth of strict anaerobes (Dubos, 1920). Many intestinal protozoa have been grown in vitro only in the presence of bacteria. If digested peptones are used for preparing the medium, the most obvious chemical action of the bacteria is to produce a very low oxidation-reduction potential. Measurements of the large intestine and caecum of the white rat with a vacuum tube potentiometer show an Eh value of -195 to -200 mv. This is considerably lower than is obtained in ordinary broth with very strict anaerobic methods (-60) mv). Therefore, some of the negative results obtained in attempts to grow intestinal parasites free from bacteria might be caused by too high an oxidation-reduction potential in the medium . . . (102).

The extablishment of optimum redox potential (Eh) in culture has been described by Mitchell (127):

> The Eh of the culture is determined by the competition of two rates: the rate of production of reduced components by the cells, and the rate of production of oxidized components by the oxygen which diffuses into the culture. The rate of production of reduced components is dependent upon the number of cells in the culture and upon their metabolic acitvity, while the rate of production of oxidized components is dependent upon the rate of solution of oxygen - the latter being controlled by the area of exposed surface, the amount of stirring and the diffusion coefficient of the oxygen (viscosity of the medium) and the rate of reduction of the oxygen determined by the nature and amounts of the autoxidizable materials in the medium.

When giardia were first cultured in vitro, they required

the presence of live yeast in the medium (6) and a large air space could be left in the culture vessel. With the addition of a reducing agent it was possible to establish axenic cultures. However, as noted before, giardia will not multiply in the presence of large amounts of air even with a reducing agent present. The question remains: If oxygen is toxic to giardia, why is the organism capable of oxidative metabolism?

Experimental factors affecting giardia metabolism

During this study several physical factors affecting the metabolism of giardia became apparent. It was noted that batches containing higher than average numbers of organisms did not always respire more rapidly than batches with fewer organisms (Figures 7 and 8). Likewise, the rates of glucose consumption and glycogen utilization could not always be correlated with cell number (Figure 12 and table 12). Similar phenomena have been observed in trichomonads (41, 54). Possible reasons for lowered respiratory activity in dense cell populations could be: crowding and competition for substrates, accumulation of inhibitory waste products, changes in pH and aging of the cells. It is possible that batches of organisms with high numbers were entering the stationary phase of growth where many metabolic functions may slow. Giardia were not always harvested at exactly 72 hours of incubation. Occasionally they were harvested up to 80 hours of incubation and could have been nearing the

stationary phase.

Another factor which may have influenced the yield of organism from each harvest was the serum used in the culture medium. Human serum used in this study was collected and pooled from three local serology laboratories. However such large quantities of serum were used that it was not possible to prepare a single serum pool large enough to use through the entire study. Therefore, a uniform serum composition could not be assured. Some batches may have had high antibody titers to giardia or perhaps contained other inhibitory or stimulatory substances, causing a variation in the yield and condition of the organisms.

Inadvertent damage to the organisms may alter their metabolism. The very act of harvesting may injure the cells. It was found that shaking and aeration of the protozoa had an adverse effect on oxygen consumption. The length of time the organism was out of culture medium also affected its ability to multiply and consume oxygen. In addition, an environment without the osmotic and pH buffering capacity of serum may be detrimental to the integrity of the organism.

Variations in giardial protein content may also be attributed to the above factors.

Glucose utilization

The results of the glucose oxidase, radio-lactate and autoradiographic experiments show that giardia trophozoites consume and metabolize glucose. The concentration of glucose was observed to decrease when glucose - containing Hanks" BSS was exposed to trophozoites (Fig. 12). The rate of glucose consumption was found to be about 280 µgm/hr/10^8 organisms. This value, however, varied considerably from batch to batch of organisms.

The combined data from radio-lactate and autoradiographic experiments show that giardia not only consume glucose but metabolize it to several products. After labeled glucose was exposed to trophozoites, the chromatographic spot corresponding to glucose was smaller and contained fewer counts per minute than if the glucose had not been exposed to organisms. Furthermore, the other radioactive spots which appeared on the chromatograms could be identified as compounds other than glucose. Control chromatograms using radioactive glucose not exposed to organisms but treated in the same manner as samples exposed to organisms showed that these compounds were neither contaminants nor the result of the experimental procedure.

The purpose of this study was to identify the possible major pathways of glucose metabolism in giardia. Information was obtained which will guide the further detailed study of the individual pathways. Because this was the first study on a

species of giardia, a similar organism was sought for comparison. Trichomonads are closely related to giardia and have been studied extensively. Both genera contain flagellated protozoa which have adapted to parasitic modes of existence in near anaerobic environments in vertebrate hosts.

Although it has been shown that several trichomonads remove glucose from the suspending medium (41, 38) a literature search yielded very few radiochemical studies of glucose utilization in these organisms.

A few studies using radioactive pyruvate (64), radioactive bicarbonate (174, 175) and radioactive succinate (173) have been made on T. vaginalis. These studies have shown that T. vaginalis yields CO_2 and amino acids from glucose, CO_2 and amino acids from succinate, and lactic acid from CO_2 fixation.

Several studies have shown that glucose stimulates the growth of trichomonads (38, 86). They may be criticized however, because the media employed contained serum and therefore probably contained carbohydrates other than the added glucose.

As discussed in Results section II, the effect of glucose on the growth of G. duodenalis could not be determined. Before data bearing on this question can be obtained, a simplified culture medium must be developed. It was found that the amount of glucose present in HSP-1 medium (about 1.14 mg/ml) would not be limiting aerobically. It was calculated that the average number of organisms at the end of 3 days incubation was

$4.3x10^5$ organisms/ml. If 10^8 organisms use 280 µgm of glucose/hr, it would take the organisms in culture about 950 hours to aerobically consume 1.14 mg/ml of glucose. The organisms, however, are grown anaerobically. It has been established that many cells consume glucose much faster under anaerobic conditions (85). It would be interesting to know if giardia exhibit this phenomenon.

Glucose was found to have an effect on the level of ATP in giardia trophozoites. Figure 15 shows that under both aerobic and anaerobic conditions, glucose stimulated the production of ATP, and that more ATP was produced under aerobic conditions. The amount of ATP remained approximately constant if no glucose was present under either aerobic or anaerobic conditions.

Oxygen consumption

Data obtained in this study, employing the Warburg apparatus and oxygen electrode, have shown that giardia trophozoites can consume oxygen. The average rate of endogenous oxygen consumption, as measured by the Warburg apparatus, was found to be about 80 µl/hr/10^8 organisms. The value obtained using the oxygen electrode was 170 µl/hr/10^8 organisms. This disparity may be attributable to the different manipulations of the instruments and organisms required before measurements can be taken. It takes considerably longer to prepare and equilibrate cells to be measured with the Warburg apparatus than for the

oxygen electrode. From the end of the harvest sequence to the beginning of respiratory measurements with the oxygen electrode no more that five minutes may elapse. With the Warburg apparatus fifteen to thirty minutes may elapse before readings may be taken. Consequently, cells used for oxygen electrode measurements will be much fresher and in better shape and will consume more oxygen.

Another consideration is that in the Warburg apparatus, CO_2 produced by the organism was continually obsorbed from the medium by KOH in the center well. In the oxygen monitor the CO_2 remained in solution. The presence or absence of CO_2 may influence the respiratory rate of the organism (176).

Rates of endogenous oxygen consumption found here for G. duodenalis are similar to those reported for various trichomonads. The measurements in all the studies reviewed here were obtained using the Warburg apparatus. Doran (55) reported the endogenous oxygen uptake at pH 6.4 as ranging from 91 to 184 µl O_2/hr/10^8 organisms for four strains of T. foetus, the value depending on which strain was measured. In another study, Doran (53) gave the endogenous respiration at pH 6.4 as 120 µl O_2/hr/10^8 organisms for a cecal trichomonad of swine (probably T. suis). The endogenous value of T. vaginalis as found by Wirtschafter (64) was 70 µl O_2/hr/10^8 organisms at pH 6. Suzuoki and Suzuoki (41) however, indicated that their strain of T. vaginalis consumed 100 µl O_2/hr/10^7 organisms at pH between 7 and 7.6.

Effect of glucose on oxygen consumption

Having determined the endogenous rate of oxygen consumption of giardia trophozoites, the effect of glucose on oxygen uptake was studied. Employing both the Warburg apparatus and the oxygen monitor, it was found that oxygen consumption was greater in the presence of glucose that the endogenous rate. Warburg data indicated that glucose caused a 20 per cent increase in oxygen consumption. This effect was consistent (significant at the .01 level). Addition of glucose to the medium causes a much greater stimulation of oxygen consumption in trichomonads than in giardia. One Warburg study of T. foetus lists a value of 50 per cent stimulation with 50 μM glucose (38) while another showed 97 to 143 per cent stimulation over endogenous respiration with 0.02 M added glucose (54) and several strains of T. suis showed 56 to 166 per cent stimulation, depending on the strain (38, 53). The stimulation observed in T. vaginalis was 135 per cent with 0.02 M glucose (41).

Thus, giardia have an endogenous respiration similar to that of trichomonads but a much weaker ability to use glucose oxidatively.

The high endogenous rate of oxygen consumption, and low stimulatory effect of glucose suggested the presence of an intracellular polysaccharide pool. Experiments to determine if this was true indicated that polysaccharide was present and did, in fact, disappear in organisms held in Hanks' BSS

without glucose. The amount varied considerably from batch to batch of organisms (Table 12); older cells appeared to contain more glycogen. The rate of glycogen utilization also varied from batch to batch (Table 14).

It seems likely that glycogen utilization by giardia probably caused a dilution effect on all glucose utilization studies (glucose oxidase, glucose stimulation of oxygen uptake and radio-lactate). The amount of CO_2 and lactate produced from glucose were calculated as being about 18 percent low due to this dilution. That is, if 10^8 trophozoites use 280 µgm glucose/hr (1.55 µM) and 10^8 trophozoites also use 60 µgm glycogen/hr (.33 µM based on the molecular weight of glucose) then the glycogen makes up 17.5% of the total (glucose plus glycogen) used.

End products of glucose metabolism

Data from radio-lactate experiments carried out in this research show that giardia trophozoites in long term exposure to UL glucose, metabolize glucose to CO_2 and an acid. Chromatography of giardia extracts exposed to labeled glucose indicated that these were not the only products: As much as 20 per cent of the label was identified as amino acids and phosphorylated compounds. The relative proportions of the products varied and in some cases not all of the label was recovered. However, half of the label was usually converted to

CO_2 and acid (Table 22), while about 20 per cent was converted to other products and about 30 per cent remained unchanged (Table 23).

Possible reasons for the failure to recover 100 per cent of the label in some experiments include the production of a volatile substance (such as acetate, ethanol or formate) that evaporated from the chromatogram during drying, the absorption of label to containers, the incomplete release of CO_2 from the incubation fluid, the escape of CO_2 into the air upon opening the incubation tubes, the presence of large areas of low radioactivity not detected on autoradiograms, and physical errors in pipetting samples. The acid product of giardia metabolism is volatile on chromatograms but was accounted for in radio-lactate measurements.

The acid product of giardial metabolism was presumed to be lactic acid. Co-chromatography of incubation fluid and lactic acid served to confirm this. Subsequent studies of this organism, however, have not shown the presence of lactic dehydrogenase in the organism (189). Lindmark, studing the human giardium has also been unable to detect this enzyme (188). Lindmark claims that the end products of giardia metabolism are CO_2, acetate and ethanol. The acetate and CO_2 are produced in the same approximate ratio as the "lactate" and CO_2 ratios found here for G. duodenalis. Ethanol was produced

in very small amounts.

It is possible that the acid end product of G. duodenalis is acetate. Acetate would travel in the same region as lactate in the chromatography system used. The difficulty with this is that the radioactivity detected in the incubation fluid of the radio-lactate experiments should be lactate. According to Barker and Summerson (165), acetate and ethanol are both removed by the copper-calcium treatment used.

A possible explanation of this enigma may be that the method used is measuring radioactive acetaldehyde. This compound is the last step before ethanol or acetate in glycolysis. Acetaldehyde is not removed by the copper-calcium treatment.

One would not expect a high concentration of an intermiate, especially one as toxic as acetaldehyde, to be present in a cell. Perhaps this strain of giardia excrete acetaldehyde as a waste product. Another possibility is that the addition of trichlosoacetic acid to the incubation mixture may have converted acetate to acetaldehyde. This seems unlikly as the conversion of a carboxylic acid to an aldehyde is not easy and the chemical conditions do not appear appropriate (190).

Further study of this question is certainly in order. For the purposes of this thesis, however, the acid end product of giardial metabolism will be referred to as lactate.

The few comparable radiochemical studies of trichomonads

available were described earlier. Two describe the end products of radioactive carbon dioxide fixation in T. vaginalis (174,175). Lactate was found to be the only radioactive end product. The papers can be criticized for poor technique. The other study reported the incorporation of label from either UL glucose or labeled succinate into CO_2 and amino acids. No labeled lactate was found (173).

Most of the studies of end products of trichomonad metabolism have employed standard chemical assay methods. T. vaginalis, for example, has been shown to produce CO_2, lactate, malate, and hydrogen (36,39,52). T. foetus produces CO_2 lactate, succinate, pyruvate, hydrogen and methane (38,41,51,53,55). Pentatrichomonas gallinarum produces succinate, lactate and pyruvate (38), T. batrachorum produces lactate (54) and T. suis produces lactate, succinate, pyruvate and hydrogen (38). This list may not be complete but gives an idea of the kinds of products that trichomonads produce from glucose.

NADH oxidation

Giardia are capable of oxidizing NADH, but this study did not define the specific method by which the organism accomplishes this important task.

NAD has been called the universal carrier of electrons in the cell. It is the usual acceptor for those dehydrogenases involved in passing electrons to oxygen in the mainstream of respiration (130). The metabolism of a cell is so dependent

on maintaining the proper ratio of NAD/NADH in order to drive its reactions that some means of oxidizing NADH or its counterparts must always be available. There is a limited amount of NAD in a cell. If NADH cannot be oxidized, the metabolism of the cell would quickly come to a halt.

In cells growing aerobically the oxidation of NADH is uaually carried out through the classic electron transport system (ETS). NADH derived from the TCA cycle in the mitochondria can be transferred directly to the ETS. On the other hand, NADH produced in the cytosol from such processes as glycolysis cannot penetrate the mitochondrion. It may be oxidezed however by one of the so-called mitochondrial shuttle systems. An example of this is the ∝ glycerophosphate-dihydroxyacetone phosphate shuttle. In this system NADH is oxidized by reaction with dihydroxyacetone-P to form NAD and ∝glycerophosphate (∝GP). The∝GP penetrates the mitochondrion and is oxidized. The dihydroxyacetone-P thus formed leaves the mitochondrion and may accept electrons from another external NADH and repeat the cycle. The NADH formed inside the mitochondrion when the∝GP is oxidized to dihydroxyacetone-P is passed on to the ETS (129). There was no attempt in this research to determine if such a shuttle system exists. There is conflicting evidence as to the presence or absence of mitochondria in giardia (12,161,162,188), but it seems most likly that giardia

do not contain this organelle.

Other methods of aerobic oxidation of NADH are known and parasites in particular are endowed with a varitey of these. Certain trypanosomes, for example, possess a non-mitochondrial ∝GP oxidase system for oxidizing NADH, with oxygen as the terminal acceptor. It is comprised of two coupled flavoprotein enzymes which perform the following reactions:

1. ∝ GP + O_2 ⟶ dihydroxyacetone P + H_2O_2
2. ∝ GP + H_2O_2 → dihydroxyacetone P + H_2O

Sum: ∝GP + $\frac{1}{2}O_2$ ⟶ dihydroxyacetone P + H_2O

The enzyme for reaction 1 is an aerobic dehydrogenase and for reaction 2 a substrate-specific peroxidase. This oxidase system is coupled to a soluble ∝GP dehydrogenase:

3. ∝ GP + $\frac{1}{2}O_2$ ⟶ dihydroxyacetone P + H_2O
4. dihydroxyacetone P + NADH + H^+ ∝ GP + NAD^+

Sum: NADH + H^+ + $\frac{1}{2}O_2$ ⟶ NAD^+ + H_2O

This system is said to be unique to trypanosomes and is insensitive to cyanide, aside, amytal and antimycin A. This suggests electron transfer in these trypanosomes is not mediated by cytochromes (112,105).

The oxidation of NADH via a number of flavoproteins to hydrogen peroxide has been observed in certain other trypanosomes (112) and in trichomonads (113). In fact flavoproteins play a major part in electron transport in these organisms (113).

Although some parasitic protozoa, such as the trypanosomes, live in environments rich in oxygen (e.g., the blood stream), many parasites live in places such as the intestinal tract or vagina, lacking or nearly devoid of oxygen. For these organisms, whose energy is derived mainly from anaerobic metabolism, use of a conventional TCA and ETS to oxidize NADH is prohibited or greatly limited. The methods of NADH oxidation in these organisms are almost as varied as the number of different organisms. Parasites in general produce a great variety of end-products from carbohydrate metabolism. Many of these compounds are the products of dehydrogenase reactions and serve as electron or hydrogen sinks, thereby permitting the reoxidation of coenzymes (114). Such products as lactic acid, propionic acid, butyric acid and even hydrogen are encountered.

The formation of lactate from pyruvate with concomitant oxidation of NADH is perhaps the most common method of NADH oxidation found in anaerobic organisms. It is a product found in many parasites. The formation of alcohol from pyruvate, although it occurs in E. histolytica (189), does not commonly occur in parasites (23).

An interesting method of anaerobic oxidation of NADH has been noted in certain hemoflagellates and Tetrahymena (126,124). In these organisms succinate is a major product of anaerobic metabolism. Experiments have led investigators to propose that CO_2 is fixed by pyruvate to yield malate, and

succinate is formed via fumarate by the reversal of part of the TCA cycle with accompaning NADH oxidation. This is apparently similar to a pathway described by Wood and Werkman for bacteria (144).

Another method of NADH oxidation, which is particularly attractive in view of the end products of giardia metabolism, has been suggested (164,166). During the study of citrate synthesis in E. coli and Streptococcas faecalis it was found that the oxidation of pyruvate may proceed in either of two ways. In the presence of orthophosphate and transacetylase the dismutation of 2 moles of pyruvate to acetyl phosphate, CO_2 and lactic acid occurs (S. faecalis). If, however, condensing enzyme and oxaloacetate are present citrate instead of acetyl phosphate is formed (E. coli).

The acetylphosphate producing system is dependent on the presence of orthophosphate, NAD and diphosphothiamine. The system is thought to proceed as follows:

$$\text{Pyruvate} + \text{NAD} + \text{CoA} \longrightarrow \text{Acetyl -CoA} + CO_2 + \text{NADH}$$

then

$$\text{Acetyl CoA} + \text{Pi} \xrightarrow{\text{transacetylase}} \text{Acetyl phosphate} + \text{CoA}$$

In addition the organisms contain lactic dehydrogenase which can oxidize pyruvate in the following manner:

$$\text{Pyruvate} + \text{NADH} \longrightarrow \text{Lactate} + \text{NAD}$$

If the two systems are considered together the following

dismutation has occured:

1) Pyruvate + Pi + NAD $\longrightarrow$ Acetyle phosphate + CO_2 + NADH

2) Pyruvate + NADH $\longrightarrow$ Lactate + NAD

3) Sum 2 Pyruvate + Pi $\longrightarrow$ Acetyl Phosphate + CO_2 + Lactate

With this scheme equal amounts of lactate and CO_2 can be formed without the presence of a TCA cycle or cytochrome system.

Evidence for cytochromes in giardia is conflicting at best. A spectrophotometric study suggested the presence of cytochromes b and c (Fig. 16). The quantity of these substances was rather low. A newer, more sensitive method, done at lower temperatures exists for obtaining cytochrome spectra (177,178) and was subsequently used by Meyer (191). No cytochromes were detected in these giardia samples.

It was found that cyanide, an inhibitor of cytochrome oxidase, inhibited CO_2 production from glucose at 10^{-1} and 10^{-2}M concentrations (Table 17). These are rather high concentrations compared to those needed to inhibit mammalian systems (less than 10^{-4}M in many cases)(88). Most mammalian studies have been done on cell-free homogenates. It is possible that giardia are relatively impermiable to cyanide; intracellular concentrations may have been much lower.

When lower concentrations of cyanide (10^{-3} to 10^{-4}M) were uses, stimulation of CO_2 and lactate production and oxygen consumption were observed (Tables 10 and 17). The phenomenon of cyanide-stimulated respiration has been encountered in trypanosomes and

and trichomonads (89). The mechanism is unknown but several explanations have been suggested. One possibility is that when cyanide combines with iron porphyrins of certain organisms the cyanide-hemochromogens have redox potentials favoring hydrogen transport, thus allowing increased oxidation of substrate (89).

Another proposal is that since CN- can bind keto groups it may bind pyruvate. This would remove a necessary substrate for further oxidative metabolism and thereby stimulate glycolysis to produce more pyruvate and consequently more NADH which would cause an increase in electron transport and oxygen uptake (148). It was not explained why this should occur in parasites and not in mammalian cells.

Cyanide is known to inhibit catalase (179). If giardia contain catalase the effect of cyanide might be a reflection of the failure of catalase to breakdown peroxide. In the Warburg studies, if catalase is decomposing H_2O_2 to oxygen and water, and the oxygen was reevolved this would not be apparent. The inhibition of peroxide breakdown by cyanide would thereby cause an apparent increase in oxygen consumption.

Still another possibility is that giardia contain a branched electron transport system. If cyanide inhibits the conventional ETS this may release constraints on the branch system which may reduce oxygen at a higher rate. Certain trypanosomes are known to have branched electron transport systems parts of which are cyanide insensitive (112,180). Control mechanisms on these branched

systems have not been studied.

Cyanide and 8-hydroxyquinolin mimic the effect of 2, 4-dinitrophenol, an uncoupler of oxidative phosphorylation. All 3 of these substances stimulate CO_2 production from glucose in giardia and stimulate the uptake of oxygen. Whether the mechanism of action is the same for all three substances is not known. 8-hydroxyquinolin may inhibit a large number of metallo-enzymes (90). Since giardia probably do not have mitochondria or cytochromes, the mode of action of these inhibitors remains unclear in this organism.

Amino acid syntheses

The ability of giardia to synthesize many different amino acids from glucose indicates the presence of certain basic metabolic pathways. If the organism relied entirely upon its host for amino acids it is possible to envision the organism lacking some of the fundamental enzyme systems that provide carbon skeletons such as the TCA or pentose cycle. Giardia, however, when exposed to UL glucose for a long period of time (30 minutes) produced numerous amino acids via their own metabolic machinery.

None of the amino acids produces were completely identified but chromatographic analysis indicated some of them may be lysine, histidine, glutamic acid, serine, threonine, glycine, hydroxyproline, cysteine, tryptophane, aspartic acid and others.

The production of aspartate, threonine, lysine or hydroxyproline points to the presence of a functional TCA cycle. Aspartate is produced from oxalacetate (91) and in turn can be metabolized to threonine (92). Through transamination, aspartate may be involved in the production of glutamate from alpha ketoglutarate (93). Hydroxyproline is synthesized from glutamate in several steps (94). The synthesis of lysine also depends on the initial presence of TCA intermediates or products (95).

Serine, glycine and cysteine may be derived ultimately form the EMP pathway (96). Histidine synthesis may involve intermediates of pureine biosynthesis or the pentose phosphate pathway (97,18). Tryptophan synthesis however, requires intermediates of both glycolysis (phosphoenol pyruvate) and the pentose phosphate pathway (erythrose-4-phosphate) (99).

Thus it can be seen that the amino acids produced from glucose by giardia may implicate three of the major pathways of glucose metabolism.

Metabolic control and regulation

The study of metabolic regulation has not been extensive for parasites. The primary direction of research into parasite physiology in the last thirty years has been to establish the existence of the major pathways in the medically important parasites. The thrust has been to look for metabolic systems that are different from the host's and which are suseptable to

drugs that do not harm the host. Only recently has there been an increase of literature on the study of individual enzymes and their properties. Parasite physiologists have finally started to isolate, purify and characterize individual enzymes from parasites, still mainly in hope of finding differences from the host that are medically applicable. Nonetheless this has added to the pool of information on regulatory mechanisms.

It is not surprizing that this information has been so long in coming. Consider the physical problems involved in obtaining enough contaminent-free parasites to isolate sufficient quantities of the enzymes. Parasitic protozoa are quite often much more difficult to culture than bacteria and are not easily obtainable in large quantities from the host. Growing large quantities of parasitic protozoa for enzymatic extraction is even more challenging and often quite expensive. Many enzymatic studies have awaited the development of large scale culture methods or the development of sensitive, micro-enzyme assays.

It is apparent that these problems are being overcome as more and more studies on mechanisms of metabolic control in parasites are being published. For example lactic dehydrogenase in T. gallinae has been shown to regulate the size of the pool of glycolytic intermediates (132).

The malic enzyme has beenshown to have a regulatory role in several parasites (106,132,140). In T. gallinae there may be evidence of an interlocking regulatory mechanism with lactic

dehydrogenase (115). The malic enzyme is the only TCA enzyme found in this other ise TCA-less organism. The malic enzyme also apparently plays a regulatory role in C. fasiculata (106). The inhibitation of this enzyme by products of glucose metabolism (oxalactate, acetyl-CoA and oxalate) "...appeared to be integral to the aerobic fermentative process...". It provides a mechanism which avoids the recycling of pyruvate and allows the organism to excrete succinic acid as an end product. This ability to use an intermediate product of metabolism as a hydrogen acceptor has been suggested as chemical adaptability lacking in most eucaryotes (106). Malic enzyme has been suggested as a regulator of NAD/NADH, malate/pyruvate ratios and cytoplasmic pH by several workers (115).

Pyruvate kinase, (PK), an enzyme with known regulatory functions in organisms that utilize glucose and store glucose as polymers, apparently does not act in this capacity in C. fasiculata (107). The enzyme was found to be an allosteric protein but the only activator found was H^+. It is not subject to the feed-forward activation by fructose diphosphate or any of the well known activators and inhibitors of other PK systems. Pyruvate kinase is important in gluconeogenisis in most organisms, and since C. fasiculata does not carry out gluconeogenisis this is given as one explanation for its non-regulatory role in this protozoan.

It seems apparent that the nature of regulatory mechansims in parasites do not differ radically from those in bacterial and

mammalian cells. However the details of control may be very different in response to the type of environment the parasite has had to adapt to. Many parasites are aerobic fermentors and their control mechanisms will reflect this. An organism that has no TCA cycle or cytochrome system but still utilizes oxygen may have very different ways of controlling ADP/ATP and NAD/NADH ratios in the cell.

Aerobic respiration is certainly more efficient in yielding carbon skeletons and ATP, but in most environments where parasites live many of the necessary carbon skeletons are already provided by the host. Consequently the organism gets along quite well without oxygen and the attendant oxidative pathways and their control mechanisms. If the organism has the necessary synthetic pathways, the presence of the end products of those pathways provided by the host, could act as feedback control substances turning the pathways off.

Parasites have been described as "metabolic opportunists" (114). Where concerted efforts have been made to establish the existence of major pathways of metabolisms they have often been found. Low levels of enzymes may merely reflect inhibition of the pathway due to the environment the organism has been living in (e.g. low levels of TCA enzymes under anaerobic conditions). However, when a new environment becomes available the parasite is often found capable of functioning in it. This may be a measure of how well a parasite is adapted to its host and the parasitic

mode of living. Organisms that are evolutionarily recent parasites may retain much or all of its original oxidative machinery. An organism that has been a parasite for a very long time may have lost certain metabolic sequences and be so dependent upon its host for these functions that it cannot adapt well to new environments. For example, consider the dependence of T. vaginalis on added catalase when grown aerobically.

Finally it might be noted that oxidative metabolism in normally anaerobic parasites may be an artifact of in vitro existence. It may play no role in the normal life of the organism; it may only reflect, a metabolic system not yet entirely lost through evolution.

Mechanisms of metabolic control were not studied as such in this work, however it is interesting to speculate on the possible effects a parasitic mode of life may have on an organism's metabolism. Many parasites spend most of their life in an environment of limiting oxygen concentration and non-limiting substrate (food, energy source). How might this effect the enzyme levels of energy producing systems? Of synthetic pathways? What control or constraint does the host put upon the organism? Are the metabolic control mechanisms in aerobic fermentors as varied as their end products? Is aerobic fermentation a functionally useful mechanism in parasites that normally metabolize anaerobically? or is aerobic fermentation an artifact of in vitro manipulation?

The answers to these questions may be useful in designing chemotherapeutic agents effective against specific parasites.

Pathways of glucose metabolism in giardia

Several lines of evidence indicate the presence of a typical glycolytic pathway in giardia:

a) Giardia consume glucose and glycogen, either of which are required for the pathway to function.

b) There is chromatographic evidence for the presence of phosphorylated intermediates of glycolysis in the organisms.

c) Glucose stimulates the production of ATP anaerobically.

d) Fluoride and iodoacetate, two glycolytic inhibitors, depress the production of lactate from glucose and inhibit growth of giardia.

e) Lactate is a major end product of glucose metabolism.

f) Lactate was produced equally from the 1 and 6 positions of glucose.

g) Hexokinase and aldolase have been detected in G. lamblia (188).

Because CO_2 was also an end product of glucose metabolism, giardia must posess a pathway for its production. The most obvious possibility is the TCA cycle. No direct studies were made in search of the TCA enzymes or intermediates and the evidence is conflicting for its presence.

a) More ATP was produced from glucose under aerobic that anaerobic conditions. The ratio of anaerobically to aerobically produced ATP was 1:3 (Fig. 15). This could be accounted for as 2 ATPs from glycolysis and 6 ATPs from the TCA cycle, since only one triose phosphate could be channeled into the TCA cycle if one was converted to lactate (the ratio of lactate to CO_2 produced from glucose was found to 1.2 ± 0.2).

b) It is unlikly that giardia contain cytochromes; necessary compounds to oxidize the large amounts of NADH produced by the TCA cycle.

c) Giardia are capable of oxidizing NADH under aerobic conditions.

d) The electron transport inhibitors 8-OHquinolin and DNP stimulate oxygen consumption indicating the uncoupling of oxidative phosphorylation, a step integral to conventional oxidative metabolism.

e) The amino acids produced from glucose indicate the possible participation of the TCA cycle as a source of carbon skeletons.

f) There is no good evidence for the presence of mitochondria in giardia. These organelles are the usual repository of enzymes of the TCA cycle and the ETS.

g) Lindmark could find no citrate synthase, isocitrate

dehydrogenase, succinate dehydrogenase or fumarate hydratase in G. lamblia. Nor did intermediates of the TCA cycle stimulate giardial respiration (188).

Carbon dioxide and lactate are produced from glucose from both the 6 and the 1 position.

Studies using C-1 and C-6 labeled glucose showed that CO_2 was not produced equally form C-1 and C-6 glucose. More CO_2 was produced from the C-1 position than the C-6 position. This could indicate the pentose phosphate pathway as an alternative method of glucose metabolism (87). Also, CO_2 and lactate production were not equally inhibited by fluoride. At 10^{-3} and 10^{-4}M, fluoride actually stimulated CO_2 production from the 1 position. This might be expected since fluoride inhibits enolase, an enzyme far down the glycolytic pathway. Thus glycolysis could be inhibited and force glucose metabolism through the pathway which branches off the glycolytic pathway at glucose-6-phosphate, far above enolase.

Future studies of giardia metabolism

When the metabolism of an organism is first being studied, the possibility for future studies are almost unlimited. However, certain basic elements of giardia metabolism still need to be established before the more esoteric aspects are investigated.

A mass culture method of obtaining large quantities of organisms must soon be an important part of giardia research. This will be

necessary because the need to extract, purify and characterize enzymes of the organism has become pressing.

It is important to establish the presence of the individual enzymes of the glycolytic pathway to confirm the data presented in this study. It is even more important to establish or disprove the presence of TCA enzymes and a conventional electron transport system and their relationship to the NADH oxidizing system of giardia. Proving the presence or absence of a TCA cycle and cytochrome system will help clear the controversy about the existence of mitochondria in giardia. The studies by Lindmark (188) subsequent to this study, are well on the way to answering these questions.

If giardia do not have a conventional oxidative metabolism, the nature of their oxidative merabolism must be sought. A search for enzymes of the pentose pathway should be made. Likewise, it is important to discover if the consumption of oxygen is truly of any importance to the organism in vitro or in vivo.

Since giardia grow best under anaerobic conditions it is essential that some of the experiments carried out in this study be repeated while the organism is under anaerobic conditions. For example, the metabolism of labeled glucose under anaerobic conditions could be studied using the radiolactate technique. Likewise glucose consumption and amino acid synthesis under anaerobic conditions should be compared with the parallel experiments

under aerobic conditions.

Trichomonads contain organelles called hydrogenosomes which are apparently the site of terminal electron transport in these cytochromless organisms. Under anaerobic conditions they produce hydrogen. The hydrogenosome is the site of a number of enzymes that are sensitive to oxygen. Here the enzymes are kept in a reduced state. These organelles are also responsible for activating metronidazole, the only known drug that is truly effective against trichomonads. Giardia are also sensitive to metronidazole. It would be of great interest to know if giardia also posess hydrogenosome-like organelles responsible for the activation of metronidazole.

These organelles may be the key to understanding the toxic effect of oxygen on giardia and trichomonads. Hydrogenosomes may, for example, provide adequate enzymatic machinery (catalase, superoxide dismutase etc.) to detoxify the low amount of oxygen encountered in their normal environment but not when exposed to the greater quantities encountered in aerobic culture vessels. Lindmark has found that G. lamblia does have SOD but that the enzymes of carbohydrate and energy metabolism are non-sedimetable (188). This implys the lack of a compartmentalized system like that of the hydrogenosome. Weimbach, on the other hand found that respiratory enzymes in G. lamblia were particle associated (192). This descrepancy may be due to the length of time the giardia homogenates were centrifuged. Both investigators believe,

however, that the electron transport mechanism in giardia involves flavins and a ferridoxin-like compound.

Another area of giardia research that is of increasing importance is the physiology of the cyst. There has been recent increased awareness of the importance of giardia as a cause of "hikers diarrhea" and as a possible problem for water treatment plants (181,142). The infective form of giardia is the cyst, the physiology of which is almost totally unknown (182).

SUMMARY AND CONCLUSIONS

It would be appropriate to reconsider the questions posed in the Introduction:

A. Do giardia trophozoites consume oxygen? Yes; the endogenous rate of oxygen consumption of these organisms was found to be comparable to that of certain trichomonads.

B. Do giardia use glucose? Yes; giardia have been shown to be capable of consuming and metabolizing glucose. It was also found that giardia contain and consume intracellular polysaccharide.

C. Is giardial growth stimulated by glucose? The results of experiments designed to answer this question were inconclusive.

D. Is giardial oxygen uptake stimulated by glucose? Yes, by about 20%. Glycogen may cause a dilution effect of about 18%.

E. What are the end products of aerobic glucose metabolism in giardia? Lactate and CO_2 appear to be the major end products of aerobic glucose metabolism. Other products of glucose metabolism, such as amino acids and phosphorylated compounds, are probably related to intermediary metabolism.

F. Does glucose affect the level of ATP in giardia? Yes; glucose stimulates the production of ATP both aerobically and anaerobically.

G. Do giardia have a glycolytic pathway? It appears that Giardia probably do contain a glycolytic pathway. Studies with C-1 and C-6 labeled glucose indicate a typical EMP type of metabolism.

Preliminary work by Lindmark (188) and others indicate the presence of glycolytic enzymes.

H. Do giardia contain cytochromes? Giardia probably do not contain cytochromes.

I. Can giardia oxidize NADH? Yes. The mechanism is not known.

J. What effects do metabolic inhibitors have on giardia? It was found that glycolytic and ETS inhibitors affected giardia metabolism at certain concentrations. In addition, DNP, an uncoupler of oxidative phosphorylation, stimulated oxygen consumption and CO_2 production. At certain concentrations cyanide also stimulated oxygen consumption and CO_2 and lactate production.

From this study giardia appear to be organisms, with a facultative type of metabolism, that have adapted to an environment of low oxygen tension and redox potential. It is assumed that the organism has a glycolytic pathway. There is also evidence for the participation of the pentose cycle. The phenomenon of cyanide-stimulated respiration indicates that electron transport in the organism is not typical. Indeed, this appears to be the most interesting physiological aspect of this organism to pursue at the present time.

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APPENDIX A

Hanks' Ballanced Salt Solution

20 X Hanks' BSS

Solution A - 20X

NaCl	160 gm
KCl	8 gm
$MgSO_4 \cdot 7H_2O$	2 gm
$MgCl_2 \cdot 6H_2O$	2 gm

Dissolve in about 800 ml distilled H_2O each in turn. Then add:

$CaCl_2$	2.8 gm

that has been dissolved in 100 ml distilled H_2O.

Mix well and bring to 1000 ml with distilled H_2O.

Solution B - 20X

$Na_2HPO_4 \cdot 7H_2O$	1.8 gm
KH_2PO4	1.2 gm
Dextrose (Glucose)	20.0 gm
0.5% Phenol Red (0.4 gm NaPhenol red)	80.0 ml

Dissolve each in turn in about 800 ml distilled H_2O, then bring to 1000 ml with distilled H_2O.

These solutions may be sterilized at 10 psi for 10 minutes or by filtration. The unsterilized solutions may be stored frozen. It is inadvisable to mix the two solutions at 20X concentration as a precipitate may form. If glucose is left out of the solution, large quantities of 1 x Hanks' may be

stored at room temperature in carboys (a 10% glucose solution may be kept frozen or refrigerated; for use add 0.5 ml of 10% glucose for every 100 ml of Hanks' needed.) For work where spectrophotometry may be involved it is advisable to omit the phenol red indicator.

APPENDIX B

Partial analysis of BBL Phytone peptone

From form #CMI-1,2 M, 5/63 published by Baltimore Biological Laboratories

Source	Soymeal
Hydrolysis	Papiac
Total nitrogen	9.2 %
Amino nitrogen	1.8 %
NaCl	4.4 %
Ca	0.05%
Fe	0.02%
K	3.99%
Mg	9.19%
P	0.38%
S	0.39%
Carbohydrate	37 %
Amino acids %	
Arginine	4.6
Aspartic Acid	5.8
Cystine	0.5
Glycine	2.8
Glutamic acid	9.3
Histidine	1.6
Isoleucine	2.5

Leucine	3.2
Lysine	3.6
Methionine	0.6
Phenylalanine	3.6
Proline	3.4
Threonine	1.8
Tryptophan	0.7
Tyrosine	1.9
Valine	2.0
Vitamins µg/mg	
Biotin	0.35
Choline	3.05
Cyanocobalamin	0.00115
Folic acid	0.81
Niacin	33.0
Pantothenic acid	7.6
Pyridoxine	4.0
Riboflavin	4.3
Thiamin	1.9
Para aminobenzoic acid	5.4

APPENDIX C

Preparation of reagents for Lowry protein determination (73).

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Reagent B: 0.5 % $CuSO_4 \cdot 5H_2O$ in 1% Na or K tartrate

Reagent C: (alkaline copper solution): Mix 50 ml of reagent A with 1 ml of reagent B. Discard after 1 day.

Reagent D: (dilute Folin reagent): Dilute the Folin reagent to make it 1 N in acid. The Folin reagent is available commercially in 2N acid form, from Hartman-Leddon Co., Philadelphia.

APPENDIX D

1. Scintillation fluid for counting $^{14}C-CO_2$

2,5 - Diphenyloxazole (PPO)	0.50 gm
1,4 - bis 2-(5-phenlyloxazoly) - Benzene: Phenyl-oxazolyplphenyl-oxazolylphenyl)(POPOP)	0.01 gm
Toluene	100.00 ml

2. Scintillation fluid for counting ^{14}C-lactate (in aqueous solution).

Scintillation cocktail (POPOP-PPO-Toluene)	700 ml
Absolute methanol	300 ml

Ten ml will take up 0.1 ml of aqueous solution

APPENDIX E

Chromatographic color reagents

1. Methyl red reagent for organic acids (169)

   a) Borate buffer 0.3 M

      A: Boric acid 12.40 gm/l

      B: Sodium tetraborate · $10H_2O$ 19.05 gm/l

      For pH 8 use 700 ml A, 300 ml B and dilute to appropriate strength for use (81).

   b) Methyl red reagent

      Methyl red 0.03 gm

      0.05 N Borate buffer pH 8 100. ml

      Organic acids appear as pink to red spots on a white background. Sensitivity of spray is 1.3 µgm of organic acid.

2. Hanes and Isherwood reagent for phosphorylated compounds (168)

   60% perchloric acid 5 ml

   4% ammonium molybdate 25 ml

   1N hydrocloric acid 10 ml

   Distilled water 60 ml

   After spraying, dry chromatogram at 85 C. Then expose paper to ultra violet radiation from a General Electric germicidal lamp (25 microwatts of 2537 Å radiation/cm^2

at 10 cm for 10 min). Organic phosphate compounds appear as blue spots.

3. Ninhydrin spray reagent for amino acids (170)

Ninhydrin	0.25 gm
Acetic acid (conc.)	4.00 ml
Acetone	96.00 ml

Note: acetic acid is added only when the last solvent system used on the chromatogram is above pH 6.