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PROTEIN GLYCONEOGENESIS IN TETRAHYMENA PYRIFORMIS

by

Ewa F. Wajnberg

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York

Winter, 1981

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

by

Ewa F. Wajnberg

PROTEIN GLYCONEOGENESIS IN TETRAHYMENA PYRIFORMIS

Thesis Advisor:

James F. Hogg

Well aerated shallow cultures of Tetrahymena pyriformis, strain GL, grown in proteose peptone/liver extract medium (PPLE) and in the exponential phase of growth, responded to heat shock treatment by the synthesis of additional glycogen.

When washed cell suspensions obtained from such cultures were shaken in the air, the glycogen content of the cells increased significantly during the incubation time (4 hours). With casein hydrolysate added to the cells, the extra glycogen synthesized by the cells was on average about three-fold higher than the endogenous increase. Under these experimental conditions, acetate had no effect on glycogen production.

A series of amino acid substrates was subsequently tested for glyconeogenesis. Among amino acids tested the best stimulant of glyconeogenesis was L-proline, but L-threonine, L-asparagine and L-leucine were also effective. L-methionine caused strong inhibition of the endogenous glyconeogenesis.

In an attempt to overcome a permeability barrier some dipeptides were tested for glyconeogenesis. Proline dipeptides with N-terminal

proline were equal to or better than the C-terminal proline dipeptides. Prolyl-valine and valyl-proline both stimulated glyconeogenesis efficiently with prolyl-valine being a slightly better substrate. Prolyl-phenylalanine stimulated glycogen synthesis efficiently. In contrast phenylalanyl-proline was a poor substrate for glyconeogenesis. The efficient dipeptide substrates (pro-val, val-pro, pro-phe) stimulated glyconeogenesis better than the mixtures of the corresponding amino acids. The use of dipeptides caused increased utilization of L-valine and L-phenylalanine by the protozoan cells and thus these two amino acids must be added to the list of the efficient substrates.

Prolyl-methionine added to the cell suspension exhibited inhibitory action that overcame the stimulatory effect of proline. However, when a mixture of proline and methionine was used, the inhibitory effect on glyconeogenesis of methionine disappeared.

Among lipid substrates tested, Tween 40 stimulated glyconeogenesis significantly. Tween 80 and lecithin had no effect on the glycogen content of the cells.

The stimulation of glyconeogenesis by L-proline is accompanied by a significant increase of NH_3 production. However, careful quantitative experiments proved that the increase cannot be accounted for by L-proline disappearance only. The incorporation of label from [U-^{14}C]-L-proline into the glycogen fraction was much lower than the value predicted from the balance study.

The conversion of proline to glutamate was confirmed by the chromatography of the protein hydrolysate of washed cells incubated

for 4 hours with radioactive proline. In the hydrolysates, labelled glutamate and aspartate could be detected, that summed up to about 50% of the total radioactivity present.

The alcohol extract of the cells contained glutamate, aspartate and asparagine, that accounted for 4.5% of total radioactivity available to the cells. Radioactive glutamine was not detected inside the cells. The incubation medium contained glutamine, asparagine, aspartate and glutamate, that accounted for 17.9% of the total radioactivity available. Thus during proline degradation amino acids accumulate and are excreted to the incubation medium. A possible mechanism for the stimulatory action of proline is suggested.

The stimulation of glyconeogenesis caused by leucine was accompanied by an increased ammonia production. The incorporation of label into the glycogen fraction from [U-^{14}C]-L-leucine was in agreement with the value predicted from the balance studies. Thus this ketogenic amino acid appears to be glucogenic in Tetrahymena. When washed cells were incubated with a mixture of L-proline and L-leucine, both the glycogen and ammonia increments were exactly additive.

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Prof. James Hogg, my advisor and mentor, has decisively influenced both my biochemical training and the preparation of this thesis. Throughout the years of my work under his supervision I came to value and respect the virtues of his mind and character and above all his wisdom in matters of academia and the world at large. In this competitive world, Dr. Hogg's incessant stress of basic biochemical problems and honest scientific work was a welcome example of how the work of a scientist can and should be pursued. For all that and for the kindness and warm friendship he has shown me and my family I am deeply grateful.

To Dr. Daniel Sverdlik, who spent lengthy hours helping me to produce the computer-made graphs and who is a good and valued friend, I want to express special thanks and appreciation.

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GENERAL INTRODUCTION

A. Historical review: protein glyconeogenesis in Tetrahymena pyriformis

The ciliated protozoan, Tetrahymena pyriformis, has been often used to study glyconeogenesis and its regulation because of the large amount of glycogen synthesized during the growth of the organism (1,2). This unicellular eukaryote resembles a mammalian cell with respect to organellar content (e.g. it contains mitochondria and peroxisomes) while at the same time its cultures can be manipulated with relative ease.

The regulation of lipid glyconeogenesis in Tetrahymena pyriformis has been studied quite extensively (3) because of its biochemical novelty. In comparison little is known about the glyconeogenesis from protein. This study was designed to fill this gap in our knowledge of the intermediary metabolism of this ciliated protozoan.

An amino acid-stimulated glyconeogenesis has never been clearly demonstrated in Tetrahymena. The major reason for this is the poor rate of uptake of the single amino acids by the ciliate (4,5). It is known, however, that the protozoan can grow well in a medium containing only proteins or polypeptides (1) and that during the time of growth a significant amount of glycogen is synthesized (up to 22% of the dry weight of the organism). Thus protein glyconeogenesis in these cells appears to be quite significant to their metabolism.

Two early papers (2,6) suggest that amino acid-stimulated gluconeogenesis can occur in Tetrahymena. Wagner (2) reported that washed stationary phase cells accumulated much glycogen upon aerobic incubation with shaking. During this period of gluconeogenesis both protein and lipid decreased in sufficient amount to provide the carbons for the glycogen formation. Subsequently, Wagner (2) showed that cells of Tetrahymena pyriformis can convert acetate (and endogenous phospholipids) to glycogen. Furthermore, in the strain studied (E) added acetate eliminated the decrease in protein, thereby inducing Wagner to study lipid glyconeogenesis--a novel metabolic pathway. The fact that there was no stimulatory effect on glyconeogenesis when washed cells were fed a mix of 11 essential amino acids (L-amino acid mix: methionine, arginine, threonine, tryptophan, valine, isoleucine, leucine, phenylalanine, histidine, lysine and serine) excluded amino acids from the study. Wagner did note, however, that phenylalanine alone had a small but significant effect.

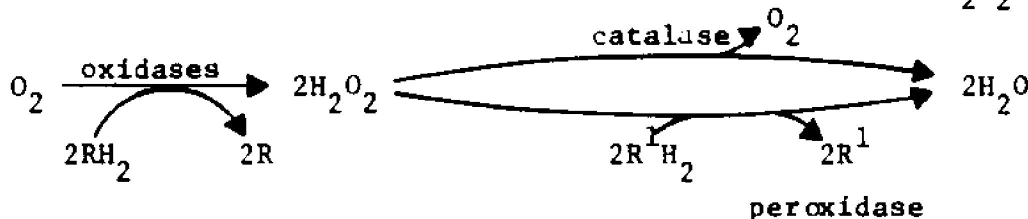
Later Scherbaum and Levy (6) demonstrated that during the development of synchronous cell division by repeated heat shocks in a proteose-peptone medium, glycogen content of the cells (strain GL) increased four-fold. This indicated that glycogen was formed at a good rate, presumably from the breakdown products of the peptides in the medium. The conditions Scherbaum and Levy used in their experiments (6) were used as a starting point for the experiments described in this study.

The importance of glycogen in higher animals is evidenced during starvation when it is the first emergency source of energy. The same

does not occur in Tetrahymena, where glycogen is not a main energy source for the organism--in washed cell suspensions the glycogen level actually increases (3,4).

It has been established that a major factor in protozoal glyconeogenesis is the glyoxylate cycle (4), which channels fats into glycogen. Adult mammalian tissues do not possess this anaplerotic cycle and thus the main noncarbohydrate precursor for glucose synthesis in those tissues must be amino acids.

The work of Hogg and Kornberg (7) was the first to associate the glyoxylate cycle with peroxisomes. This organelle, sometimes also called glyoxysome or microbody, is a large (0.5 microns) organelle bounded by a single membrane and containing a granular matrix. The name is based upon the organelle's characteristic content of flavoprotein oxidases and catalase, which make it a site of H_2O_2 metabolism:



From the time of its discovery, the peroxisome has been associated with gluconeogenesis. In his first review of peroxisomes, De Duve (8) points out that, in mammals this organelle is found only in tissues capable of gluconeogenesis. The postulated role of mammalian peroxisomes in the process remains to be established. However, in Protozoa (3,7), yeast (9), plant seedling (10) and green leaves (11), various peroxisomal enzymes contribute to a gluconeogenic pathway (glyoxylate cycle or glycolate pathway). Very recently toad bladder

mucosa has been found to contain the necessary enzymatic activities required for a glyoxylate cycle (12). In addition Jones (13) has presented evidence for the presence of the glyoxylate cycle in the liver of a fetal guinea pig. The last two observations strongly suggest that the currently accepted dogma--the absence of the glyoxylate cycle in mammalian tissues (14)--should be reexamined.

In Tetrahymena, two key enzymes in the peroxisome: isocitrate lyase and malate synthase, enable the ciliate to produce glycogen from acetate via the glyoxylate cycle. Figure 1 represents the interrelationship between peroxisome and mitochondrion in this process (3,15).

Hogg and Kornberg (7) showed that cells grown on a synthetic medium low in the ketogenic amino acids lacked the key enzymes of the glyoxylate bypass, isocitrate lyase and malate synthase. Addition of acetate to this medium improved growth and induced the formation of both isocitrate lyase and malate synthase at high levels. In terms of growth improvement, however, glucose had much more effect than acetate. On the basis of these findings, it can be concluded that a classical pathway of protein gluconeogenesis has a minor contribution to glyconeogenesis in this organism because the gluconeogenic amino acids of the medium apparently could not supply glucose in sufficient quantity for a good growth rate.

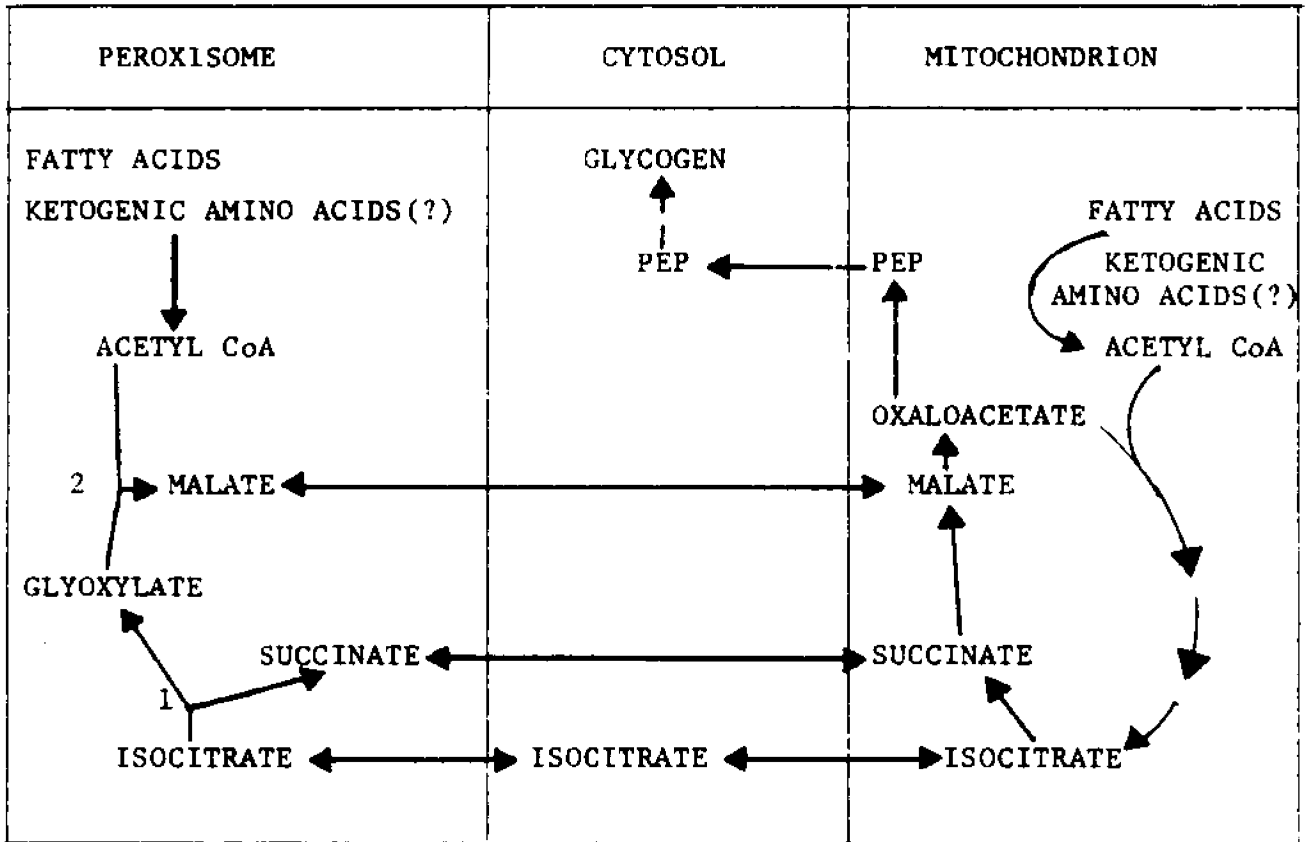
In order to study protein gluconeogenesis in the protozoan, it is necessary to find conditions where protein is preferred to lipid as the substrate for gluconeogenesis. Strain GL of Tetrahymena pyriformis (now called T. furgasoni) meets this condition. Suspensions of washed cells of this organism, grown in a proteose-peptone, liver

Figure 1: Interdependence between peroxisomes and mitochondria in the glyoxylate cycle of *Tetrahymena pyriformis*.

Enzymes:

1. Isocitrate lyase
2. Malate synthase

Figure 1



extract medium with good aeration (6,16) did not incorporate radioactive carbon from acetate into glycogen to a significant extent, whether harvested at the exponential or the stationary phase of growth. In cells from such cultures, isocitrate lyase and malate synthase are at high level and increase further as the cultures enter the stationary phase. Nevertheless, even though the enzyme levels are sufficient, extracellular acetate did not support glyconeogenesis (3,15). Therefore it seems quite likely that under these conditions glycogen is being synthesized from amino acids.

Study of the amino acid-stimulated glyconeogenesis is complicated by the fact that amino acids are often major excretory products of the organism. For example, Reynolds (17) found that the main metabolites released to the medium during incubations of washed cell suspensions were alanine, glutamic acid and glycine. Tetrahymena do not have the urea cycle (18) and thus, the end products of nitrogen catabolism in the ciliate are ammonia and, as mentioned above, certain amino acids (4). In addition, Tetrahymena cells have an unusually high content of free amino acids and peptides (4% of the dry weight)(19). It was the purpose of this work to find suitable conditions for good glyconeogenesis from protein and, once such conditions were established, to search for effective glycogenic amino acids. Once discovered, studies of their metabolism in Tetrahymena should determine their mode of utilization in glyconeogenesis.

Even though all glucogenic amino acids can be incorporated in large part into glycogen in mammals, some tissues have a decisive preference for certain amino acids. In the human liver, alanine represents the

most important amino acid precursor for gluconeogenesis (2) . In perfused rat liver, among amino acids the best substrates for gluconeogenesis were L-serine, L-alanine, L-proline, L-asparagine and L-glutamine (21). Valine and isoleucine were not converted to glucose in significant degree. Similarly, Tetrahymena cells utilize certain amino acids better than others. For example, the oxidation of amino acids by Tetrahymena pyriformis (strain W) as studied by Roth et al. (22) showed good utilization of the following amino acids: L-phenylalanine, L-tyrosine, L-cysteine, D-cysteine, L-proline, L-isoleucine, L-leucine and D-leucine. These amino acids stimulated the O_2 consumption from 10 to 50%. Proteose-peptone doubled the respiratory rate of the ciliate (4).

Mavrides et al. (23,24,25) studied the regulation of glyconeogenesis from amino acids in Tetrahymena pyriformis, concentrating on the phenylalanine catabolizing system and glyconeogenesis from this amino acid. Using 3 days old cultures of Tetrahymena (strain W) grown with or without acetate, they followed the flow of radioactivity from amino acids to glycogen in washed cell suspensions. In the absence of acetate in the growth medium, the best incorporation into glycogen was obtained for L-tyrosine, L-isoleucine and L-leucine. Growing the cells in the presence of acetate suppressed the incorporation of ^{14}C from amino acids into glycogen (with the exception of aspartate). The researchers established that Tetrahymena contain a phenylalanine catabolizing system identical to the one present in mammalian liver. However in this study the amino acids were supplied only at a tracer level and thus, we can not know if they can produce a net increase of glycogen in the cells. In other words, the data presented by Mavrides et al.

may be irrelevant to net, i.e., physiologically significant protein glyconeogenesis.

Elson et al. (26) studied amino acid glyconeogenesis following the inhibition of protein synthesis in Tetrahymena pyriformis (strain E). When cycloheximide was added to log-phase cultures, the decrease in protein synthesis was accompanied by a sharp increase in the incorporation of 2-^{14}C-acetate and of U-^{14}C-algal protein hydrolysate into glycogen. When cultures were incubated with radioactive amino acids for 4 hours, out of the 17 amino acids tested, the following ones exhibited good incorporation of label into glycogen: L-arginine, L-lysine, L-threonine, L-tyrosine, L-isoleucine and L-serine. Addition of cycloheximide increased the incorporation significantly in every amino acid studied.

In mammals amino acids are divided into glucogenic (converted into glycolytic or TCA cycle intermediates) and ketogenic (converted into ketone bodies: acetoacetate, β-OH-butyrate and acetone). This distinction, however, may not apply to the ciliate. Under the conditions of an active glyoxylate cycle, amino acids ketogenic in mammals can become glucogenic in Tetrahymena. The following diagram (Diagram 1) summarizes the fates of amino acids in Tetrahymena pyriformis (4). The diagram reflects the present knowledge of amino acid metabolism in the ciliate. It is based mainly on growth requirements and nutrient sparing effects and in some cases is quite incomplete. In case of uncertainty, I assumed a similar pathway to the one in mammalian liver but lack of evidence is indicated by a question mark. It should be noted from the diagram that pyruvate carboxylase, a key factor in liver gluconeogenesis, is absent in Tetrahymena pyriformis (27).

Diagram 1: Amino acid metabolism in *Tetrahymena pyriformis*.

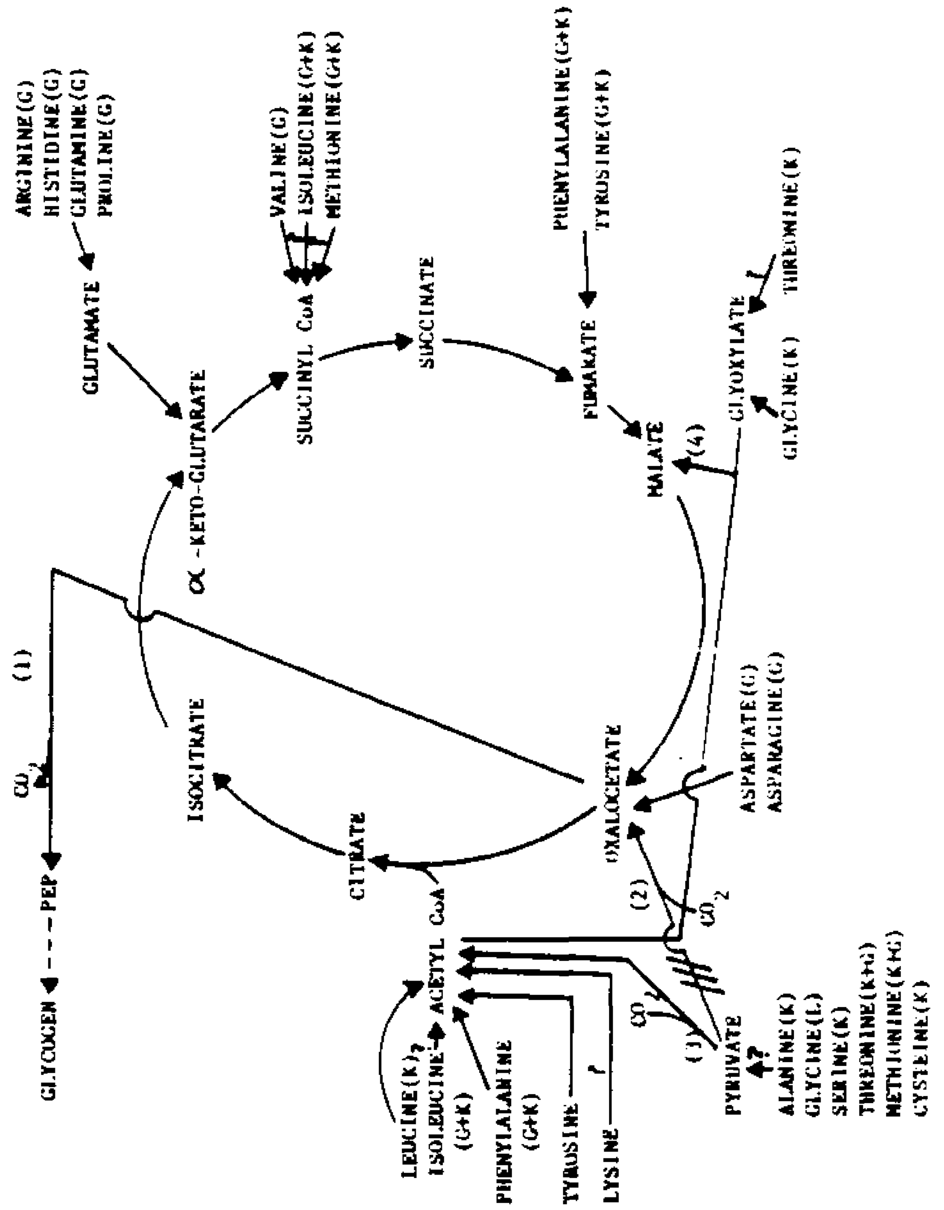
Designations:

K - Ketogenic

G - Glucogenic

1. Phophoenolpyruvate carboxykinase
2. Pyruvate carboxylase
3. Pyruvate dehydrogenase
4. Malate synthase

Diagram 1



Amino acids may stimulate glyconeogenesis either by contributing carbon atoms for the glycogen synthesis or by participation in some regulatory mechanism. Williamson et al. (28) reported that, when rat liver is perfused with alanine plus oleate, the glucose production is doubled over alanine alone. They attributed this increase in glucose production in part to the elevated NADH/NAD ratio maintained in the cytosol during enhanced fatty acid oxidation. Until recently fatty acid oxidation was believed to occur entirely in the mitochondrion. In 1969, however, Cooper and Beevers (10) reported that certain plant seedlings contain a β-oxidative pathway starting with an acyl-CoA oxidase. This finding was recently confirmed by Hryb and Hogg (29) for Tetrahymena and by Lazarow (30) and Hryb et al. (31) for the rat liver. Subsequently it was shown by Hashimoto (32) that liver peroxisomes have the entire set of enzymes needed to oxidize fatty acids. Peroxisomes are surrounded by a membrane that has a rather high permeability to low molecular weight compounds (4). Even though there exists a certain permeability barrier in peroxisomes (e.g. latency of NADP-isocitrate dehydrogenase (33)), it is known that NADH formed inside peroxisomes diffuses out to the cytosol. Peroxisomal β-oxidation of fatty acids, therefore, could produce an increased NADH level in the cytosol, since during β-oxidation NADH is produced at one step. β-oxidation in peroxisomes also will release acetyl-CoA which, in liver, may activate pyruvate carboxylase. Alternatively amino acid catabolizing enzymes, whose action results in NADH production, could enhance glyconeogenesis if located in cytosol or in the peroxisome.

The last part of this introduction will summarize the experimental approach used in the following study.

B. Experimental approach

In order to study protein glyconeogenesis in *Tetrahymena pyriformis*, this experimental framework had to be obtained:

1. The development of a technique for protein-stimulated glyconeogenesis in washed cell suspensions;
2. Identification of the effective amino acid and peptide substrates;
3. Determination of the incorporation of ^{14}C from amino acids (added at substrate level) into glycogen as verification of net glyconeogenesis results;
4. Elucidation of the metabolic pathways in protein glyconeogenesis by means of isolating intermediate products of ^{14}C-labeled amino acid substrates.

EXPERIMENTAL PROCEDURES

A. Methods

1. Cultivation and harvesting of protozoan cells

Tetrahymena pyriformis, strain GL,* was cultivated in a medium containing 2% proteose-peptone, 0.05% liver extract and 0.1% dipotassium phosphate. In later experiments 0.1% of L-Proline was added to the medium, since it appeared to improve the reproducibility of the experiment with proline. The medium was made sterile by autoclaving 15#/in^2 for 15 minutes. It will be referred to as PPLE. Stock cultures were kept in 19 mm screw cap test tubes containing 10 ml of the medium. Mass cultures were grown in flat cylindrical flasks, 7 inches in diameter provided with a side neck. Each flask contained 100 ml of the medium. The inoculum contained 2 ml of the stock culture. A typical growth curve is shown on Figure 2. The cultures were shaken (60 RPM) at 25° for about 40 hours or as desired. Cultures at this time are in the late log phase. The number of the dividing cells at this point of growth is significant, as can be observed under the phase contrast microscope.

To obtain washed cell suspension, the cells were harvested from 100 ml batches with a modified plankton centrifuge (34), washed in the same volume of Ryley's modification of Krebs-Ringer phosphate medium (RRP (35)), and resuspended to a final concentration of 1-2% (v/v), as

---

*Tetrahymena furgasoni, ATCC 3006.

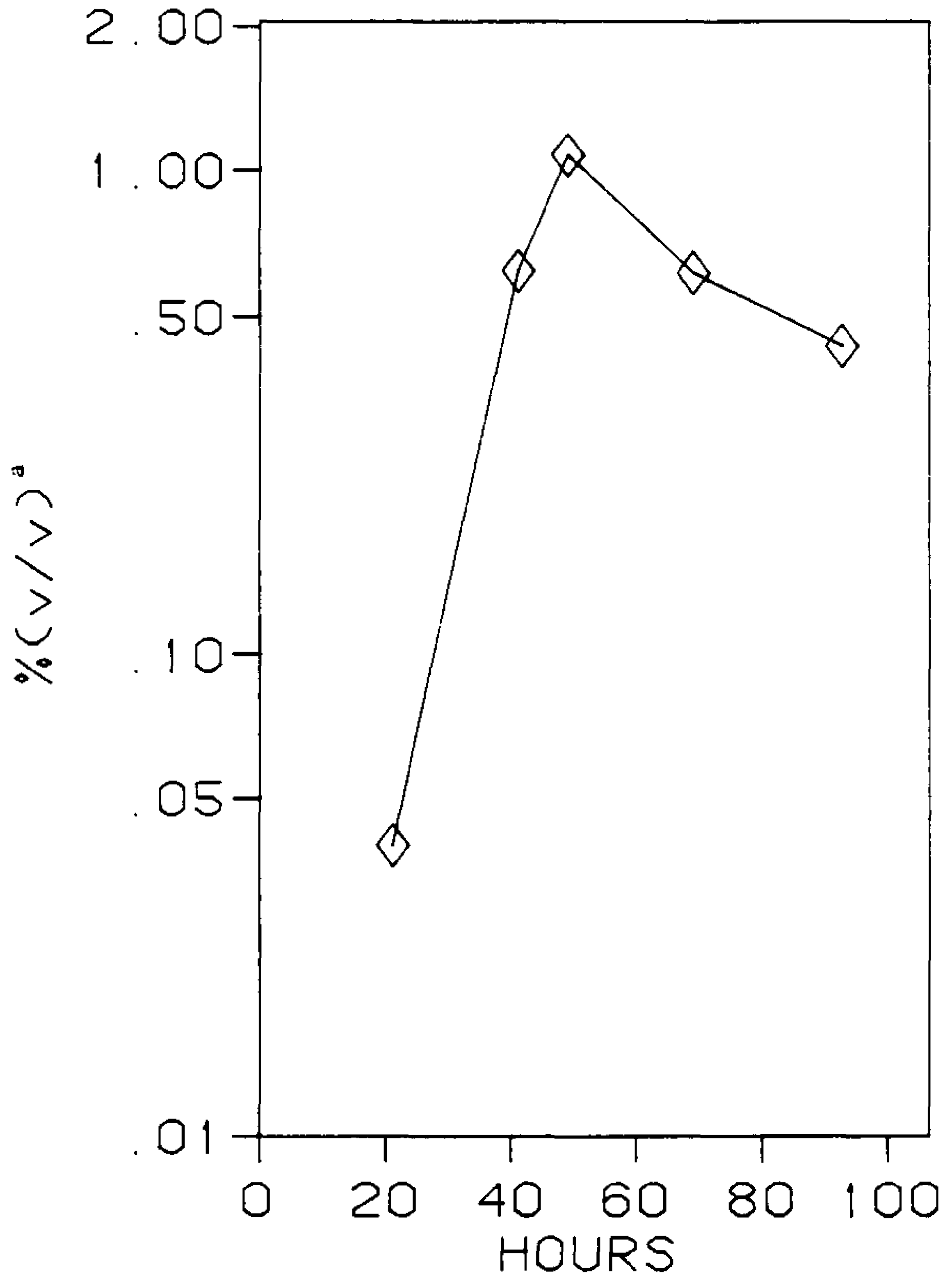
Figure 2: Growth Curve of *Tetrahymena pyriformis* strain GL in well aerated PPLE cultures.

Composition of the medium (PPLE).

proteose peptone	2%
liver extract	0.05%
dipotassium phosphate	0.1%

a. %(v/v)--percent cells in the incubation medium, as determined by centrifugal packing (1000g) in a Constable protein tube.

Figure 2



determined by centrifugal packing (1000g) in a Constable protein tube. The composition of RRP buffer is given below:

COMPOSITION OF RRP		
Salt Added	Final Concentration (mM)	
NaCl	46	
KCl	2	
$MgSO_4 x 7H_2O$	1	
KH_2PO_4	2.4	TOTAL 12
Na_2HPO_4	9.6	pH=7.4

2. <u>Heat shock treatment procedure in exponentially growing cultures of Tetrahymena pyriformis, strain GL</u>

The procedure used was based on the work of Scherbaum and Levy (6). Cultures from the log phase were transferred to 250 ml flasks, 25 ml of culture per flask. Those were exposed to 5-6 hours of the heat shock treatment: they were incubated alternately in 29° (optimum temperature) and 34° (shock temperature) for 0.5 hours each for a total period of 5-6 hours, while shaken at 90 RPM. This is a method to rapidly arrest cell division, and upon release from the treatment synchronized division of the cells occurs.

3. <u>Glyconeogenesis in washed cell suspensions</u>

Washed cells were incubated with or without added substrate in 25 ml Erlenmeyer flasks, containing 2.0 ml of the 2% cell suspension and shaken at 100 RPM in temperature of 29. The incubation time in

most experiments was 4 hours.

Casein hydrolysate used as a substrate for glyconeogenesis was made by preparing a 5.75% suspension and sterilizing it, stoppered with a cotton plug, for one hour at 100°. The solution was cooled prior to use. The final concentration of casein hydrolysate in the incubations was 2.7 mg/ml. Samples were taken for glycogen determination at times 0, 2 and 4 hours.

4. Glycogen determination (36)

For each determination, 2 volumes of 45% KOH were added to one volume of cell culture or suspension. This mixture was boiled for 30 minutes. The insoluble precipitates were removed by centrifugation (1000g x 10min), washed with 1.0 ml of water and both supernates were pooled.

In experiments involving radioactive tracers, the incubation was stopped by addition of 1.0 ml of 7% perchloric acid. The glycogen was then precipitated from the Acid Soluble Fraction (see "Incorporation of tracer" in this section, part 5).

1.2 volumes of 95% ethanol were then added to the supernates to precipitate glycogen. Induction of flocculation of glycogen was achieved by immersing the lower half of the test tube in water bath at 70° for 30 minutes, or by leaving the tubes overnight at 0°. The precipitated glycogen was centrifuged (1000g x 10min), supernate was removed and the glycogen redissolved in 2.0 ml of water. The precipitation was repeated with 2.5 ml of 95% ethanol. The second precipitate was dissolved in 1.0 ml of water in case of KOH method or 1.0 ml

of 0.05N NaOH in case of perchloric acid extraction method. The tubes were stored at 0° until use.

For the color reaction, aliquots of 0.1 and 0.2 ml of sample were taken and 10.0 ml of anthrone reagent (0.2 gms of anthrone dissolved in 80% (v/v)H_2SO_4, prepared by mixing 27.5 parts of ice-cold water with 80 parts of ice-cold concentrated H_2SO_4) was added to the aliquots. The contents wre mixed on Vortex-Genie (Fisher Scientific). Glucose was used as standard at the range of 25-100 micrograms. The standard glucose solution contained 500 micrograms of glucose in 1.0 ml of water saturated with benzoic acid. A set of standards was determined each time. An average standard curve is shown in the Appendix, Figure 1.

The samples with the reagent were heated for 10 minutes at 100°, then cooled in an iced bath and finally the Absorbance at 620 nm was determined in a Spectronic 20 colorimeter. The results are expressed as micrograms glucose/ml of cells, micromoles glucose/ml of cells or micromoles glucose/mg protein.

5. Metabolic transformation of radioactive tracers (37)

The following tracers were used in these experiments: 2-^{14}C-acetate, U-^{14}C-L-Proline and U-^{14}C-L-Leucine.

The incubations were conducted in Cavett flasks (25 ml Kontes metabolic reaction flask). Each flask received the following additions:

In the cup: 0.5 ml of 0.1N NaOH and a folded 1 x 3 cm wick of filter paper.

In the base: 2.0 ml of washed cell suspension

0.025 ml of tracer solution (initial concentration = 10 microcuries/ml)

0.05-0.1 ml of desired substrate

A flask without a tracer was run as a blank. Complete incubation mixture was placed on water bath shaker (Aquaterm Water Bath Shaker - New Brunswick Scientific), shaken at 100 RPM, in 29°. The incubations were stopped by quickly adding 1.0 ml of 7% perchloric acid. Then the flasks were restoppered and shaken for another 30 minutes to allow for complete absorption of CO_2. At this point the tracer was added to the blank.

Fractionation

A. CO_2

The caps were removed, paper wick was mixed well with the NaOH solution and portions of $Na_2C^{14}O_3$ were checked for radioactivity content. Diluted tracer solution (25 microliters of tracer in 2.47 ml of 0.1N NaOH) served as an internal standard.

B. Glycogen, Lipids, Protein, Nucleic Acids and Acid Soluble Fraction

The acidified cell suspension was sonicated in the flask for 1 minute at setting 3 on the Branson Model 140D Cell Disrupter (Heat Systems Ultrasonics) with microtip, in order to extract glycogen completely. The contents of the flasks were transferred to centrifuge tubes with two 1.0 ml washes of 2% perchloric acid. The procedure

employed to obtain glycogen, protein, lipid, nucleic acids and Acid Soluble Fraction (ASF) is depicted on Diagram 2.

The fractions obtained were subsequently assayed for radioactivity. Duplicate aliquots were plated on planchets and then dried by gentle heating under an infrared lamp. The samples were counted then on the Nuclear-Chicago Planchet Counter (low background model) for 2000 counts or 5 minutes, whichever comes first.

Alternatively, duplicate aliquots were pipetted into 10 ml of scintillation fluid (Patterson-Green) (38) and vials were counted in Scintillation Counter (Beckman 200 Liquid Scintillation System.) The pre-set probable error was 2%.

The results were calculated in the following manner:

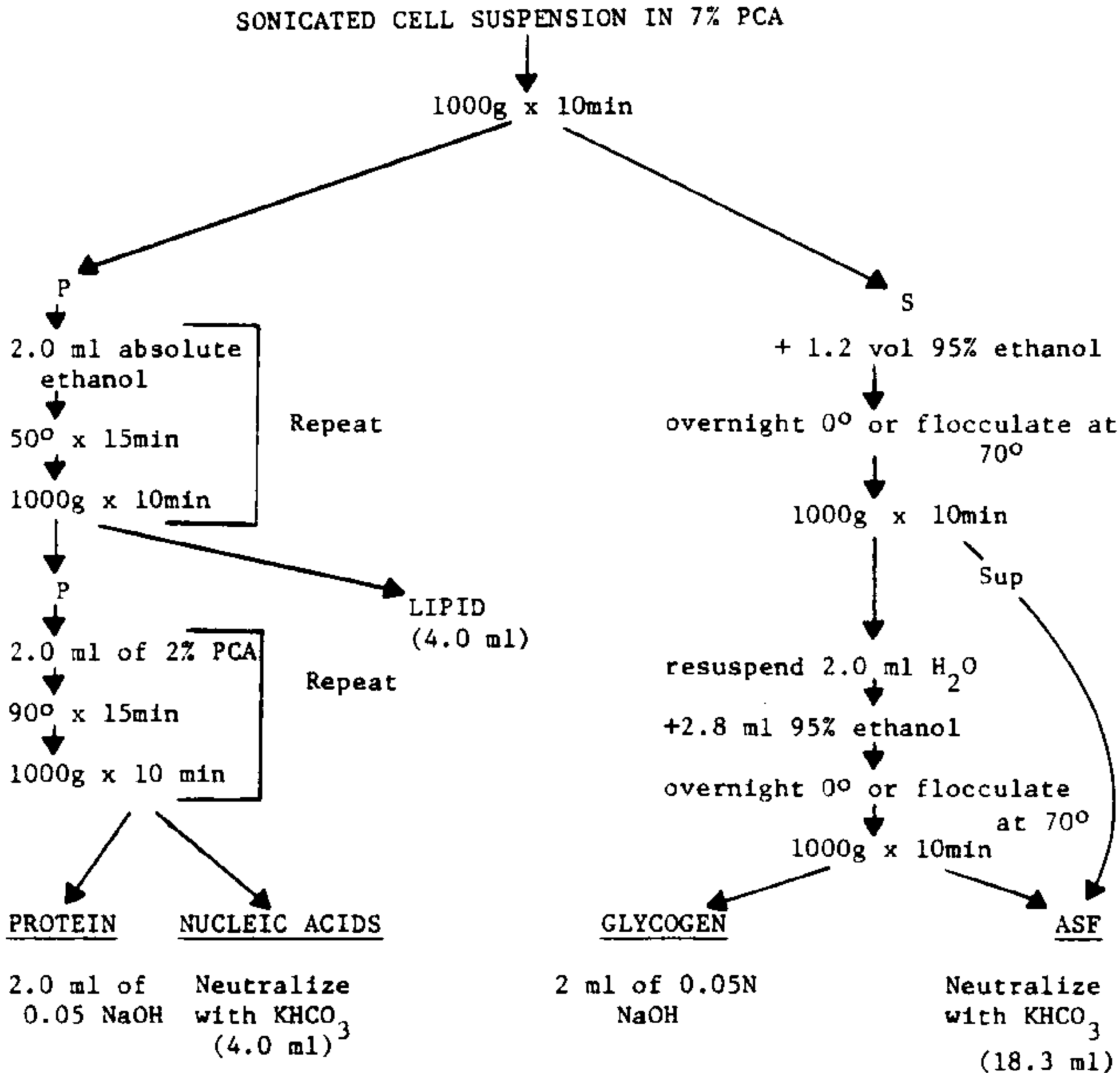
$$\frac{(\text{cpm x total volume})_{\text{FRACTION}}}{(\text{cpm x total volume})_{\text{INTERNAL STD}}} \times 100\% = \%\text{ of }^{14}\text{C contained in the Fraction}$$

6. Determination of ammonia nitrogen (39)

A 2.0 ml portion of 2% cell suspension containing suitable additions was placed in the base of the Cavett flask. 0.5 ml of 0.1N HCl was placed in the cup. The flasks were tightly stoppered and incubated with shaking (100 RPM) at 29°. At a desired time 4 ml of 45% KOH was introduced into the base and the mixture was allowed to stand tightly stoppered for 48 hours. 2 ml of cell suspension was similarly treated at the time 0. To check the recovery, 0.20 mg of N (from $(NH_4)_2SO_4$ standard) was treated in the same way at time 0. The method was proved to give 100% recovery. Glutamine and Asparagine were shown to lose one mole of ammonia upon the treatment.

Diagram 2

Procedure to obtain lipid, protein, nucleic acids, glycogen and acid soluble fraction



2.0 ml of 0.05 NaOH

Neutralize with $KHCO_3$ (4.0 ml)

2 ml of 0.05N NaOH

Neutralize with $KHCO_3$ (18.3 ml)

After 48 hours the contents of the cups containing HCl were quantitatively transferred to a large test tube, calibrated to a final volume of 25.0 mls. The contents were diluted to about 75% volume, when 2.5 ml of Nessler reagent was added. The volume was brought to 25.0 mls with water, the contents mixed and tubes were allowed to stand for 30 minutes. The color that developed was read in a Spectronic 20 at 515 nm against a reagent blank, containing water instead of an ammonia sample. Standard solutions of $(NH_4)_2SO_4$ containing 0.1 and 0.2 mg of ammonia nitrogen were analysed in the same way. Standard ammonium sulfate solution contained 1 mg N/10 ml. Figure 2 in the Appendix shows a typical standard curve obtained.

7. Uptake of L-U-^{14}C-proline and L-U-^{14}C-leucine

Flasks containing 2.0 ml of 2% cell suspension, 0.025 ml of the tracer (initial concentration: 10 microcuries/ml) and 0.050 ml of 84 mM amino acid (final concentration: 2.0 mM) were placed in the Water Bath Shaker at 29° and shaken at 100 RPM. At indicated times (including time 0) the cell suspension was transferred to a Constable protein tube. The Erlenmeyer flask was washed out with 0.5 ml of RRP and the mixture was centrifuged (setting 10(250g) x 3 minutes, International Centrifuge, size 2, model K). Supernate was decanted to another tube. The packed cells were washed in the following way. The tip of a Pasteur pipette was introduced to the bottom of a Constable protein tube containing the packed cells, and its contents (1.5 ml of RRP) rapidly released, causing resuspension of the cells. The suspension was centrifuged (setting 10 x 3 minutes) and the supernatants combined. The volume of the supernate is 4.0 ml (diluted

twice). Aliquots were counted in liquid scintillation counter (error 2%. The cell pellets were saved for alcoholic extraction (see Section 8). Some incubation flasks contained tracer at high level (0.2 ml) when radioautography was to be performed.

8. Alcoholic extraction of amino acids (40)

The packed cells obtained above (see Section 7) were resuspended in 0.5 ml of RRP and 1.5 ml of 95% ethanol was added. The ethanol extraction was achieved by heating tubes at 60° for 25 minutes. The tubes were centrifuged (1000g x 10min) and the supernate carefully decanted. This supernate contains organic acids of the cells.

9. Hydrolysis of protein (41)

The following incubations were performed: 2.0 ml of 2% cell suspension, 0.2 ml of U-^{14}C-L-Proline (10 microcuries/ml) and 0.05 ml L-Proline (84 mM). The incubations were terminated after 4 hours by pipetting in 1.0 ml of 7% perchloric acid. Subsequently the protein fraction was obtained, as stated in Section 5. The protein fraction was transferred to a 10 ml capsule with a long neck, by means of Pasteur pipette. The contents of the tube were washed with 1.0 ml of 2% perchloric acid. The protein precipitate was lyophilized to dryness in the capsules and then 1.0 ml of 6N HCl was added to the capsules. The capsules were sealed and left overnight (16-18 hours) in an oven, maintained at 100°. The resulting hydrolysate was lyophilized to dryness and dissolved in 0.25 ml of C.1N NaOH.

10. Chromatography of amino acids

Chromatography of amino acids was performed on cell supernatant (Section 7), alcohol extract (Section 8) and protein hydrolysate (Section 9). Paper chromatography was performed in water-saturated phenol (39). 5-25 microliters of sample were applied to Whatman #1 paper in small portions and dried thoroughly. The origin was checked for sufficient radioactivity prior to development. In order to count chromatograms, a manual counter (Forro Manual scanner (Geiger tube) compiled to a Nuclear-Chicago Model 151A scaler) was used. For the final identification standard amino acids were cochromatographed with the sample.

Protein hydrolysates were chromatographed by ascending chromatography in a tank of 13 x 14 inches. Because of significant content of radioactivity in the area just above the origin, it was necessary to resolve this low peak. For that purpose, the chromatogram developed once was dried and the chromatography was repeated. The paper was dried again and the chromatogram scanned for radioactive peaks after first and second development, using manual counter (Forro tube, N-C scaler). Amino acid standards were cochromatographed with the sample. The color was developed with ninhydrin spray.

In order to resolve the chromatogram in one run, descending chromatography on large sheets of Whatman #1 paper (19 x 23 inches) was performed for the cell supernatant and the alcohol extract. After the solvent reached the bottom of the paper, the paper was dried, sprayed with ninhydrin and checked for radioactivity with the manual counter.

11. Protein determination - Biuret assay (42)

The following solutions were prepared:

1. Protein standard: 5 mg of crystalline bovine serum albumin/ml. Prepared fresh.
2. Biuret reagent: 2.62 gm of $CuSO_4 \times 5H_2O$ and 9 gm of sodium potassium tartrate in 500 ml of 0.2N NaOH, add 5 gm of KI and make to 1 liter with 0.2N NaOH.

Method:

Add 3 ml of biuret reagent to 2 ml of protein solution, mix and warm at 37^oC for 10 minutes. Cool and read the absorbance at 540 nm.

B. Materials

Liver extract added to the incubation medium was Wilson's Fraction 2NF. Casein hydrolysate used as a substrate was obtained from Sigma (No. C-0626). Sodium acetate, all amino acids and peptides were obtained from Sigma.L-α-Lecithin was obtained from Calbiochem. and detergents: Tween 40 and Tween 80 from Sigma. The tracers used 2-^{14}C-Acetate and U-^{14}C-L-proline were obtained from ICN and U-^{14}C-L-leucine from Schwarz Biochem. Ninhydrin spray was obtained from Sigma.

EXPERIMENTAL RESULTS

A. Glyconeogenesis in cells from log phase cultures. Stimulation by heat treatment

The heat treatment procedure used was based on the work of Scherbaum and Levy (6) (See Methods, Section 2). In Figure 3 results of two such experiments are shown. For each experiment the glycogen content of treated and untreated cultures is shown.

As can be seen, the glycogen content of the cultures incubated at the optimum temperature more than doubled, indicating that breakdown products of the peptides from the medium (PPLE) are used up by the cells for glyconeogenesis. The heat treatment causes a significant, reproducible stimulation of the process. In these experiments, the results of Scherbaum and Levy (6) were reproduced. This was taken as a starting point in the study of protein glyconeogenesis in *Tetrahymena pyriformis*, strain GL.

B. Glyconeogenesis from casein hydrolysate

In order to obtain clearcut evidence for protein-stimulated glyconeogenesis it was necessary to use a better defined system. Therefore washed cells of *Tetrahymena pyriformis*, strain GL, were incubated with casein hydrolysate as the amino acid source. The concentration of casein hydrolysate was 2.7 mg/ml. Assuming equal contents of 20 amino acids, it results in 0.135 mg/ml of each amino acid. Taking the average M.W. of an amino acid as 100 and the average carbon

Figure 3: Effect of heat treatment on glycogen content of the cells.

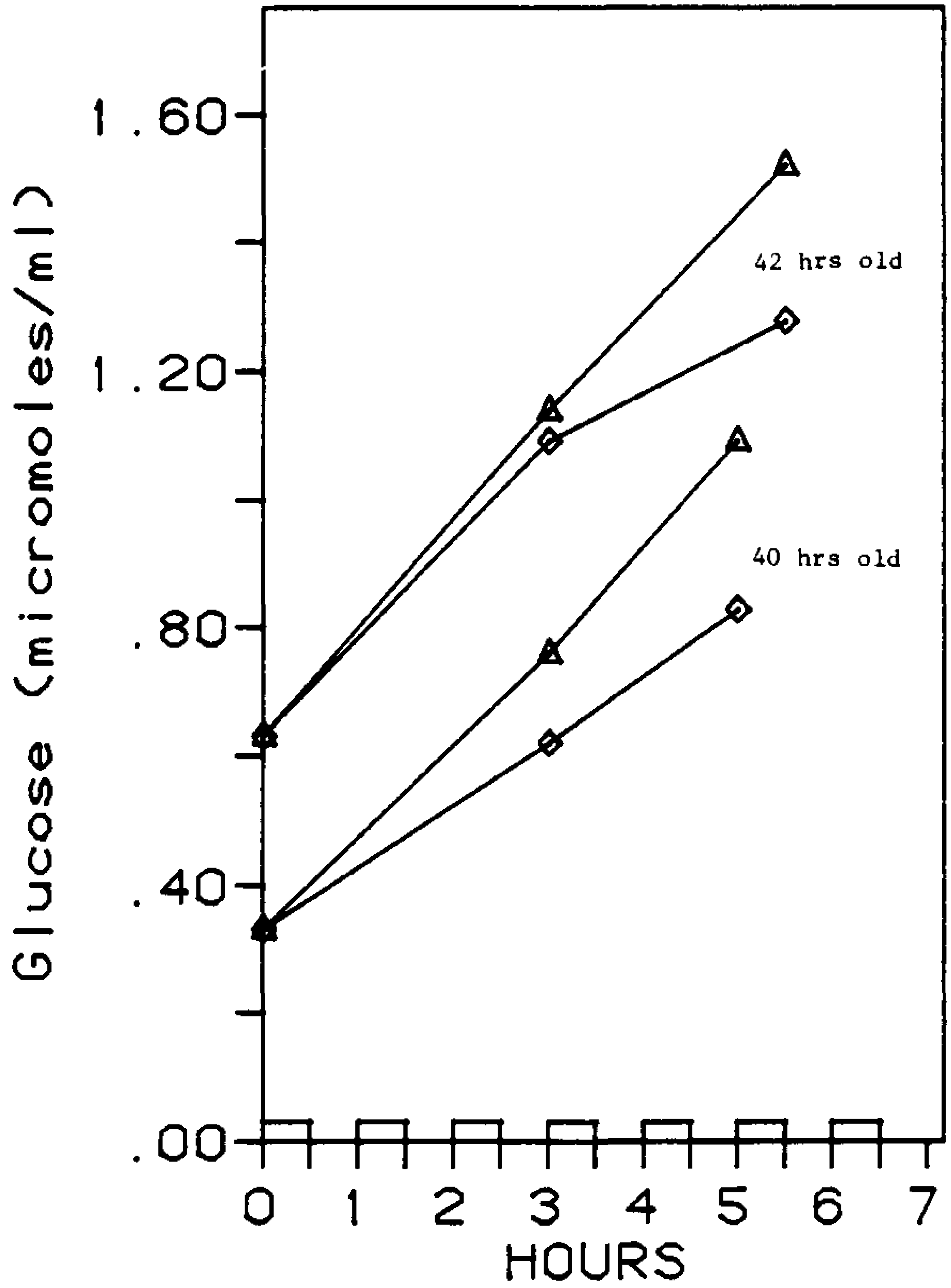
The incubations were performed as described under "Experimental Procedures," (p. 18).

Designations:

Diamonds (black) - cultures incubated at 29°C

Triangles (red) - cultures subjected to heat treatment

Figure 3



atom content of an amino acid molecule as 5, the concentration of each amino acid was 1.35 micromoles/ml or 6.75 microatoms (C)/ml. This supplies enough carbon to synthesize more than 1 micromole of glucose/ml of cell suspension.

The results of a typical experiment are shown in Figure 4. In this experiment a 37 hour old culture was harvested, washed with RRP buffer and resuspended to 1% (v/v).

When washed cells are shaken in the air, their glycogen content increases significantly during the period of incubation (3.3-fold). With casein hydrolysate added, the glycogen content increases 5.7-fold. Acetate does not increase glycogen production under these conditions, thus indicating that true protein glyconeogenesis occurs. This is therefore a suitable system to study protein glyconeogenesis in this ciliate.

The effect of casein hydrolysate is apparent between 2 and 4 hours of incubation. The lag period of the response could be explained by a slow uptake of the amino acids and peptides into the cells. The amino acids compete probably for the same transport sites in the cell membrane, making it more difficult for the effective substrates to enter the cells.

The experiments with casein hydrolysate were followed by experiments in which each of a number of amino acids was tested for glyconeogenic capacity.

C. Glyconeogenesis from amino acids

Having established that protein breakdown products seem to stimulate glycogen synthesis, it was my purpose to determine which amino acids

Figure 4: Effect of casein hydrolysate on glyconeogenesis in washed cell suspension.

Cells were harvested from 37 hour old GL/PPLE culture.

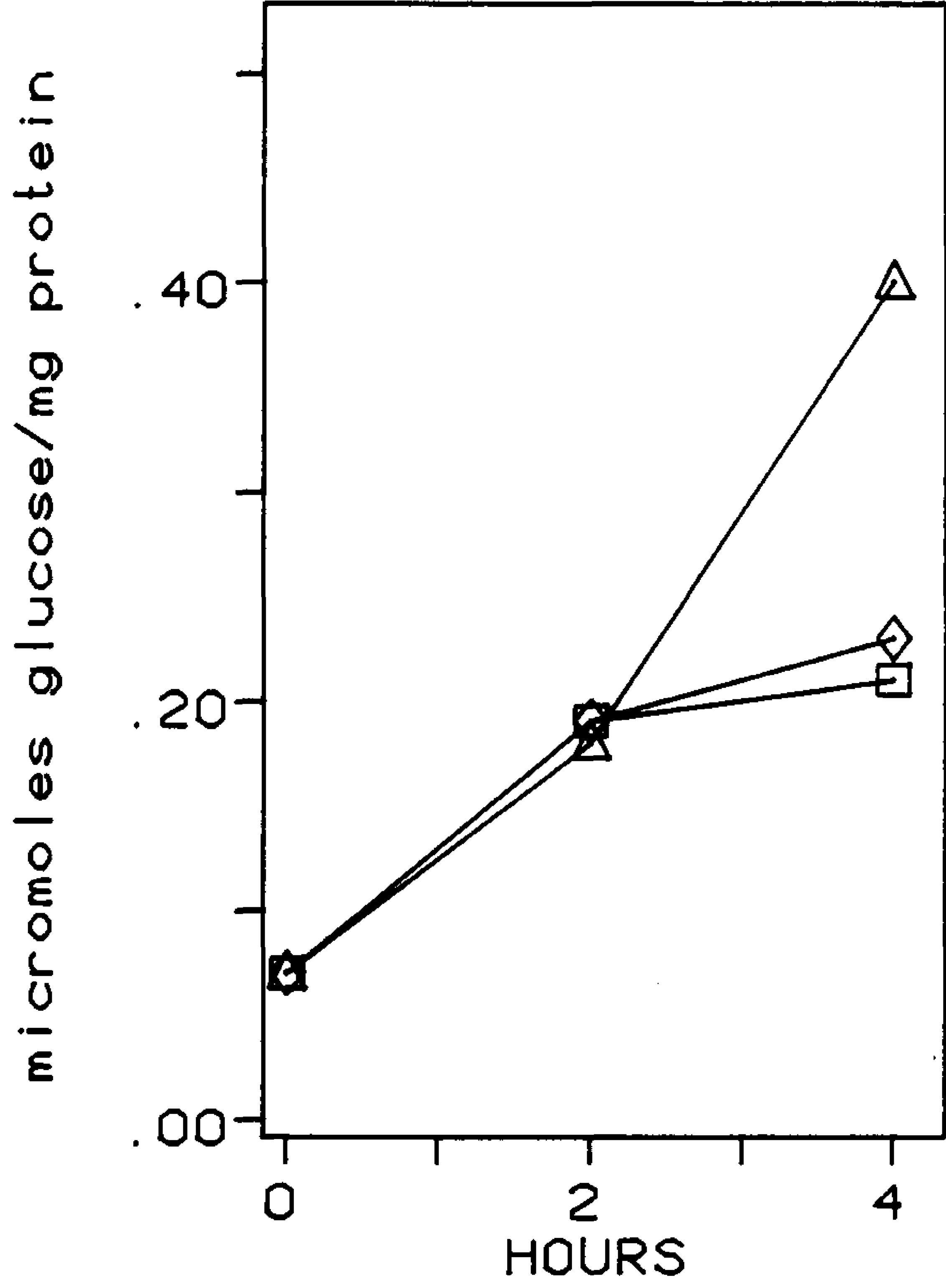
Designation:

◇ (green) - endogenous

☐ (blue) - Na Acetate (1 mg/ml)

△ (red) - casein hydrolysate (2.7 mg/ml)

Figure 4



are most effectively used by the cells for the process

The results of two sample experiments are tabulated in Table 1. For each incubation the amount of glycogen produced in four hours is reported (glycogen above 0 time). The cells used were from 37-42 hours old cultures.

In the second series of experiments, only 10 microatoms of amino acid carbon were added per ml of cell suspension to determine the efficiency of carbon flow from an amino acid to glycogen. In my best experiments, 1.0 micromole of extra glucose was synthesized per 1.0 ml of cells during the incubation time. Since there are 6 carbon atoms/glucose molecule, that is equivalent to 6 microatoms of carbon per ml. Assuming that the process occurs with 60% efficiency (which is very high), 10 microatoms of carbon/ml of a particular amino acid would be needed to produce 1.0 micromole of extra glucose. A sample calculation for L-proline is shown below.

	M.W.	# carbon atoms per molecule	# μmoles containing 10 μA/ml	Final conc.
L-proline	115	5	2	2 mM

With this procedure it was possible to calculate the apparent % efficiency with which carbon atoms from amino acids are utilized for glycogen production. In order to do so, the micromoles of glucose produced above the endogenous were calculated. This was converted to microatoms of carbon of glucose and divided by 10 microatoms of carbon available from the substrate, thus giving the % efficiency of carbon utilization in the experiment. This is a conservative calculation, since it assumes that with substrate added, endogenous glyconeogenesis

TABLE 1

Amino Acids and Glyconeogenesis in *Tetrahymena pyriformis* - Results of Two Sample Experiments[a]

GLYCOGEN (GLUCOSE) ABOVE 0 TIME (μ moles/ml)					
	EXPERIMENT 1		EXPERIMENT 2		
Conc. of a.a.	2 mg/ml		10 μ Atoms(C)/ml		
Substrate added		Factor of Stimulation[b]		Factor of Stimulation[b]	% Efficiency[c]
None (Base Line)	0.56	1.00	0.53	1.0	-
Casein hydr.	1.23	2.20	1.07	2.02	-
L-proline	1.25	2.25	1.10	2.08	34.2
L-asparagine	-	-	0.96	1.81	25.8
L-threonine	1.14	2.04	0.76	1.43	13.8
L-phenylalanine	0.82	1.46	0.74	1.40	12.6
L-alanine	0.61	1.09	0.72	1.36	11.4
L-valine	0.64	1.14	0.73	1.38	12.0
L-methionine	0.30	0.54	0.27	0.51	-[d]

a. The cells used were from 37-42 hours old cultures (T.p., GL)

b. Factor of Stimulation = $\frac{\text{Glycogen above 0 Time (Substrate)}}{\text{Glycogen above 0 Time (Endogenous)}}$

c. Calculated as described in the text

d. Cannot be calculated

still occurs at the same rate. It is possible, however, that exogenous substrate spares the endogenous one for glycogen production and that the whole increase in glycogen content of the cells is due to the added substrate. Wagner (2), however, could show that endogenous glyconeogenesis was not reduced by added glucose.

The results of the series of experiments in which amino acids were screened as the substrates for glyconeogenesis are summarized in Table 2.

It was concluded from the results shown above that among the amino acids tested, L-proline is the most effective stimulant of glyconeogenesis and its effect is comparable to the one exhibited by casein hydrolysate. L-threonine and L-asparagine consistently showed a stimulatory effect. The results for L-glutamine are inconclusive: it was a poor substrate for glycogen synthesis in three out of four experiments, but in one experiment it doubled the rate of glyconeogenesis and had an effect comparable to L-asparagine. L-phenylalanine, L-alanine, L-glutamate, L-aspartate, L-serine and L-tryptophan seemed to be poorly utilized for glycogen synthesis. L-valine, L-histidine, L-arginine, L-OH-proline and glycine showed practically no effect on glyconeogenesis, with L-OH-proline and glycine being even slightly inhibitory. Finally L-methionine showed consistently a severe inhibition of glycogen production. This toxic effect of methionine as well as the significant stimulatory effect of L-threonine, though of interest, were not pursued in this study.

The fact that the effects of L-proline and casein hydrolysate on glyconeogenesis are comparable in their magnitudes was the reason to omit casein hydrolysate from later experiments and to use just

TABLE 2

Effect of Amino Acids on Glycogen Production of

Tetrahymena pyriformis: Summary[a]

SUBSTRATE	STIMULATION FACTOR RANGE	AVERAGE STIMULATION FACTOR[b]	# OF EXPERIMENTS
Casein Hydrolysate	2.2-4.5	3.2	3
1. L-Proline	1.7-2.5	2.1	8
2. L-Threonine	1.4-2.2	1.8	4
3. L-Asparagine	1.3-2.1	1.8	6
4. L-Glutamine	0.8-2.0	1.3	4
5. L-Phenylalanine	1.1-1.8	1.4	6
6. L-Alanine	1.0-1.8	1.3	4
7. L-Glutamate	1.5	1.5	1
8. L-Aspartate	1.4	1.4	1
9. L-Serine	1.4	1.4	1
10. L-Tryptophan	1.2	1.2	1
11. L-Valine	0.7-1.4	1.0	6
12. L-Histidine	0.9-1.3	1.1	4
13. L-Arginine	0.7-1.2	1.0	4
14. L-OH-Proline	0.8	0.8	1
15. Glycine	0.7	0.7	1
16. L-Methionine	0.1-0.5	0.4	6

a, b - See a,b, Legend for Table 1, p. 35.

L-proline as the control. It is of interest to note that casein hydrolysate contains 11% of L-proline (43). In my experiments 2.7 mg/ml of casein hydrolysate was used. Out of this 0.30 mg/ml or 2.6 μm/ml was L-proline in free or peptide form. That is probably why casein hydrolysate was a lucky choice of a protein substrate for glyconeogenesis.

The effect of L-leucine on glycogen production is shown in Table 3. It can be noted that at equal concentrations (2 mM), L-leucine stimulated glyconeogenesis only slightly less than did L-proline. Both amino acids significantly stimulated NH_3 production of the cells, which is, as mentioned in the introduction, the end product of the nitrogen metabolism in Tetrahymena. This indicates a good utilization of the two substrates by the cells. The metabolic fates of these two amio acids will be discussed in detail later. L-proline is a purely glucogenic amino acid in mammals whereas L-leucine is a ketogenic one. Both stimulate glyconeogenesis in the protozoan and an attempt will be made to clarify the mechanism of the stimulatory action in each case.

It should be noted that the lack of effect of an added amino acid can be caused by a poor uptake of that amino acid by the intact cell which is indeed a problem in Tetrahymena pyriformis. It was reported by Hoffman (44) that amino acid transport in the ciliate is dependent upon sodium ion concentration in the incubation medium. My inorganic medium (RRP), in which washed cells are incubated, supplies, however, the optimal concentration (about 50 mM) of sodium ions.

TABLE 3

Effect of L-Leucine on Glyconeogenesis[a]

a. *Tetrahymena pyriformis*, GL, were harvested from 41 hours old PPLE cultures. 2%(v/v) cells suspensions were used. The incubation time was 4 hours. Amino acids were added at concentration 2.0 mM.

b. The factor of stimulation was calculated as the ratio of glycogen produced in the presence of substrate over the endogenous glycogen increase.

c. Percent utilization was calculated as the ratio of micromoles extra NH_3 produced (substrate)/ml over micromoles of substrate available/ml, times 100.

TABLE 3

Substrate Added	GLYCOGEN: Glycogen (Glucose) Above 0 Time µmoles/ml	GLYCOGEN: Glycogen (Glucose) Above Endogenous µmoles/ml	GLYCOGEN: % Efficiency	GLYCOGEN: Factor of Stimulation[b]	AMMONIA N: NH_3 µmoles/ml	AMMONIA N: NH_3 Above Endogenous µmoles/ml	AMMONIA N: % Utilization[c]
None (Base Line)	1.18	-	-	-	4.10	-	-
L-proline	1.76	0.58	34.8%	1.50	5.32	1.22	61%
L-leucine	1.59	0.41	20.5%	1.35	5.42	1.32	66%

Rasmussen (45) reported that particles in the incubation medium stimulate uptake of nutrients by the cells through induction of formation of food vacuoles in the cell. This technique was tested using two kinds of particles: polystyrene beads, 0.25 nm in diameter, and colloidal iron. The particles were incubated with cells and amino acids, but their inclusion had no effect on glycogen production of the cells. It was noted that many beads were inserted into food vacuoles of the ciliate and some were egested in vacuoles that were microscopically visible. This phenomenon was first observed by Muller (46). This lack of effect of particles is in agreement with later work of Rasmussen, who later admits that in his study of amino acid transport in *Tetrahymena pyriformis* no significant increase in the measured uptake was found by induction of food vacuoles (47). Since my attempts to improve amino acid glyconeogenesis by this procedure failed, I decided to try a variety of dipeptides as substrates for glycogen production. This was based on two reasons:

1. There are reports that peptides have a separate transport system from single amino acids and therefore might be taken up better by the cells (48).
2. One of the two amino acids (e.g. proline) could serve as a carrier and in such a way amino acid of otherwise poor uptake could get into the cell and be metabolized.

D. Peptides and glyconeogenesis

In the first series of experiments washed cells from the late log phase (about 40 hours old) were incubated with various amino acids and their dipeptides. In preliminary experiments an excess of these substrates

was added. Peptides containing L-proline were tested carefully, since L-proline alone was consistently an excellent stimulant of glyconeogenesis and it was hoped that, when attached to another amino acid, it would carry it into the cell.

For all incubations, the glycogen content/ml of cells was determined. The results are expressed, as previously, in terms of micromoles glycogen produced during the 4 hours of incubation (Δ glycogen above 0 time). For each incubation, the factor of stimulation was calculated (Δ glycogen above 0 time (substrate)   glycogen above 0 time (endogenous)), and this was the basis of comparison between different substrates. In the later experiment all substrates were added at the concentration of 10 microatoms of carbon/ml of cell suspension. The % efficiency of carbon utilization therefore could be calculated, analogously to the method described in Section 3 for single amino acids.

1. Proline dipeptides

Eight dipeptides containing L-proline were tested as substrates for glyconeogenesis: prolyl-valine, valyl-proline, prolyl-phenylalanine, phenylalanyl-proline, prolyl-alanine, alanyl-proline, prolyl-methionine and prolyl-proline. The results are summarized in Table 4A. Prolyl-methionine was omitted here and will be discussed later (Table 4B). In Table 4B, calculations are presented that allow an estimation of the utilization of the carbon of the second (non-proline) amino acid for glyconeogenesis. The contents of the table do not include phenylalanyl-proline, prolyl-proline and prolyl-methionine, because in the experiments shown these dipeptides were not tested. The

TABLE 4A

Effect of Proline Dipeptides on Glyconeogenesis[a]: Summary

SUBSTRATE	STIMULATION FACTOR RANGE	AVERAGE STIMULATION FACTOR[b]	# OF EXPERIMENTS
1. L-proline	1.7-2.5	2.1	8
2. L-valine	0.7-1.4	1.0	6
3. L-alanine	1.0-1.8	1.3	4
4. L-phenylalanine	1.1-1.8	1.4	6
5. Prolyl-valine	1.7-3.8	2.7	5
6. Valyl-proline	1.3-2.4	2.0	5
7. Prolyl-alanine	1.9	1.9	1
8. Alanyl-proline	2-2.2	2.1	2
9. Prolyl-phenylalanine	1.6-3.0	2.4	4
10. Phenylalanyl-proline	1.0-1.3	1.2	3
11. Prolyl-proline	1.1-1.2	1.2	2

a, b - See a, b, Legend for Table 1, p. 35.

substrates were supplied in concentration of 10 microatoms(C)/ml. The single amino acid efficiency in glyconeogenesis occurring was calculated as described in Section 3. The efficiency of utilization of L-proline was determined carefully at two levels:
10 microatoms(C)/ml (2 mM) and 5 microatoms(C)/ml (1 mM). The calculations were done under the assumption that with exogenous substrate added, endogenous glyconeogenesis still occurs. As can be seen (Table 4B), at both levels an average of 36.3% of the carbon content of proline appeared to be converted into glycogen.

This was used as a basis for the estimation of the efficiency of utilization of the amino acids bound to proline in dipeptides. My assumption here was that L-proline in dipeptide was utilized with the same efficiency as the free amino acid.

Each amount of dipeptide used provided 10 microatoms(C)/ml of washed cell suspension. Based on the structure of the particular amino acid it was calculated how many microatoms are donated by each of the two. Thus, in valyl-proline and prolyl-valine, 5 microatoms are donated by each amino acid. In prolyl-phenylalanine, out of 10 microatoms, 3.9 microatoms are from proline and 6.1 microatoms originate from phenylalanine. Finally in prolyl-alanine and alanyl-proline, 6.2 microatoms are the ones of proline and 3.8 microatoms are from alanine. In each case only 36% of the carbon originating from proline was converted to glucose. Therefore it is possible to calculate glucose produced in microatoms (C)/ml . This in turn is easily converted to micromoles. So, from these calculations, I can estimate how many micromoles of glucose are produced from the

TABLE 4B

Efficiency of Utilization of Substrate Carbons in Glyconeogenesis-- Comparison Between an Amino Acid and Its Proline Containing Dipeptide[a]

Substrate 10μA(C)/ml	Glycogen (Glucose) above 0 time micromoles/ml	Glycogen (Glucose) above endogenous micromoles/ml	#μA(C) contributed by each amino acid	Glycogen (Glucose) from Proline micromoles/ml	Glycogen (Glucose) from second a.a. micromoles/ml	Glycogen (Glucose) from second a.a. μA(C)/ml	% Efficiency for second a.a.
1. None	0.53	-	-	-	-	-	-
2. L-proline							
a) 10μA(C)/ml	1.10	0.57	10	-	-	3.42	Avg:
b) 5μA(C)/ml	0.85	0.32	5	-	-	1.92	36.3
3. L-valine	0.74	0.21	10	-	-	1.26	12.6
4. Pro-Val	1.10	0.57	Val: 5	0.30	0.27	1.62	32.4
5. Val-Pro	1.10	0.57	Pro: 5(36.3%:1.82)	0.30	0.27	1.62	32.4
6. L-Phe	0.74	0.21	10	-	-	1.26	12.6
7. Pro-Phe	1.60	1.07	Phe: 6.1 Pro: 3.9(36.3%:1.42)	0.24	0.83	4.98	81.6
8. L-Ala	0.71	0.18	10	-	-	1.08	10.8
9. Pro-Ala	0.99	0.46	Ala: 3.8	0.38	0.08	0.48	12.6
10. Ala-Pro	0.99	0.46	Pro: 6.2(36.3%:2.25)	0.38	0.08	0.48	12.6

a. The calculations were done as described in the text.

available proline. By subtracting that amount from the experimental value of the glycogen produced above the endogenous for a particular dipeptide, the micromoles of glucose made from the second amino acid of the dipeptide are obtained. This value is converted to the corresponding microatoms(C) glucose/ml. Since I know the number of microatoms(C)/ml available from the other amino acid, % efficiency can be calculated by comparison of these two values.

As shown in Table 4B, L-valine and L-phenylalanine are utilized much better when bound to proline (32.4% and 81.6% efficiency respectively) than when added as single amino acids (12.6%). This is a dramatic improvement, which shows that these dipeptides are indeed taken up by the cells better than the corresponding amino acids. An additional proof for a better utilization of these dipeptides is given in Table 5. In this experiment a mixture of L-valine and L-proline, and L-phenylalanine and L-proline (all at the concentration of 1.0 mM) were tested as substrates for glycogen production. The glyconeogenesis in the incubations containing those mixtures, was in both cases the same as with L-proline (1 mM) alone. This indicates that indeed the higher utilization of the dipeptides discussed above occurs due to their better uptake by the cells, followed presumably by the intracellular hydrolysis of the peptide bond. Upon these results, intracellular L-phenylalanine and L-valine must be added to the list of the efficient substrates for protein glyconeogenesis in Tetrahymena pyriformis.

L-alanine was a poor substrate either when added in free form or when bound to proline, and thus it appears not to be converted to glycogen efficiently by the organism. This is contrary to liver gluconeogenesis

TABLE 5

Lack of Stimulatory Effect of L-valine and L-phenylalanine, Administered in the Presence of 1mM Proline, on Glyconeogenesis.

Substrate	Glycogen Above 0 Time micromoles/ml
1. None	1.18
2. L-proline(1mM)	1.43
3. L-proline(2mM)	1.76
4. L-valine(1mM) + L-proline(1mM)	1.41
5. L-phenylalanine(1mM) + L-proline(1mM)	1.36

for which L-alanine is one of the most effective substrates among amino acids (20). In Tetrahymena, however, alanine is known to be excreted in considerable quantity (17).

The effectiveness of the mixed dipeptides tested seemed to depend on the position of L-proline. The most pronounced effect can be observed with phenylalanyl-proline and prolyl-phenylalanine (Table 4A). Prolyl-phenylalanine exhibited consistently a significant stimulatory effect(2.4-fold on average), that was often higher than the effect of L-proline by itself. However, phenylalanyl-proline appeared to be a very poor substrate for glyconeogenesis. It is of interest to note that there is a dramatic difference in solubilities between prolyl-phenylalanine and phenylalanyl-proline. Phenylalanyl-proline is readily soluble in RRP but prolyl-phenylalanine is practically insoluble in this buffer. The solution had to be prepared by dissolving the prolyl-phenylalanine in dilute acid and bringing the pH to 7 with dilute base. By this procedure a colloidal suspension is produced. It is possible that the fact that prolyl-phenylalanine is fed to the cells as a colloidal dispersion can in part explain the effectiveness of this substrate. Such dispersion has unique physical properties and may be more easily swallowed by the cells.

Valyl-proline and prolyl-valine are both very good stimulants of glyconeogenesis in the ciliate (Tables 4A and 4B). However, the average stimulation factor seems to be significantly higher in the case of prolyl-valine (2.7) than valyl-proline (2.0). Finally, prolyl-alanine and alanyl-proline stimulated glyconeogenesis to the same small extent, and the position of proline did not seem to matter in the case of these

dipeptides. In conclusion, proline dipeptides with N-terminal proline were equal to or better than C-terminal proline dipeptides.

In order to examine whether L-proline is utilized better for glycogen synthesis when bound to another amino acid than when added alone, prolyl-proline was tested as a substrate. Unfortunately this dipeptide appears to be a very poor substrate (Table 4A) for glyconeogenesis, perhaps because it cannot be hydrolyzed by the cells. In case of prolyl-phenylalanine (Table 4B), however, even if proline is utilized with 100% efficiency, phenylalanine still must be used with 41% efficiency to account for the glycogen increase obtained in the experiment. In summary, the use of proline dipeptides did result in a more efficient glyconeogenesis from two amino acids: L-valine and L-phenylalanine.

2. Methionine dipeptides

Three peptides containing methionine were studied: methionyl-alanine, alanyl-methionine and prolyl-methionine at the level of 10 microatoms (C)/ml of cell suspension. As mentioned before, L-methionine inhibits glyconeogenesis under these conditions. The results are summarized in Table 6.

Alanyl-methionine and methionyl-alanine exhibited an inhibition of glyconeogenesis comparable to the inhibition obtained with methionine alone (see Table 6).

Similarly, when methionine was attached to L-proline, its inhibitory action overcame the stimulatory action of L-proline. However, when a mixture of the two amino acids was added at the level of 10 microatoms(C)/ml of each, the inhibition of methionine disappeared and the glycogen production was the same as the one produced with

TABLE 6

Effect of Methionine-dipeptides on Glyconeogenesis[a]

SUBSTRATE 10 μA(C)/ml	STIMULATION FACTOR RANGE	AVERAGE STIMULATION FACTOR[b]	# OF EXPERIMENTS
1. L-Proline	2.1-2.5	2.25	3
2. L-Methionine	0.1-0.5	0.40	3
3. L-Prolyl-L-Methionine	1.2-1.7	1.45	2
4. L-Proline + L-Methionine	2.4	2.40	1
5. L-Alanine	1.0-1.4	1.20	2
6. L-Alanyl-L-Methionine	0.4	0.4	1
7. L-Methionyl-L-Alanine	0.3	0.3	1

a,b - as in Legend of Table 1, p. 35.

proline alone (Table 6). A possible explanation of such results could be a common transport system for L-proline and L-methionine that has a preference for L-proline. If such transport system is indeed there, then to see the inhibitory effect of methionine in the dipeptide prolyl-methionine, it has to be taken up by the cells and hydrolyzed inside.

3. Polypeptides

Three polypeptides were tested as substrates for glycogen synthesis: poly-L-valine, poly-L-proline and tri-L-alanine. Apparently none of these peptides was used by the cells for glycogen production because the glycogen increase was equal to the nonsubstrate control. Although *Tetrahymena pyriformis* produces extracellular proteases (4), it seems that none of these peptides could be hydrolyzed by the extracellular enzymes. Thus di-proline and polyproline appear to be equally inert.

4. DL-alanyl-DL-asparagine

Since L-asparagine was a good substrate for glyconeogenesis in *Tetrahymena pyriformis*, the dipeptide DL-alanyl-DL-asparagine was tested, even though it contains non-utilizable D-amino acids. The results are shown in Table 7.

D-alanine and D-asparagine seem to have little effect other than a slight inhibition on glycogen production. It is important to mention here that Wu et al. (49) showed that D-lysine and D-methionine are used efficiently by the cells and that D-amino acids showed no toxic effects in growth studies.

TABLE 7

Effect of DL-alanine-DL-asparagine on Glyconeogenesis[a,b]

Substrate	Ave. Stimulation Factor
L-asparagine	2.00
L-alanine	1.40
D-asparagine + L-asparagine	1.40
D-alanine + L-alanine	1.10
DL-alanine-DL-asparagine	1.95

a,b - as in Legend, Table 1, p. 35.

The results indicate that the effect of the dipeptide can be attributed to L-asparagine alone. Thus it appears that peptide bonding has at best abolished the small inhibition exerted by the D-amino acids used. L-alanine appears not to be utilized well either alone or in combination with L-asparagine. Since the substrates were added in excess, this is only a qualitative evaluation.

E. Effect of Lecithin, Tween 40 and Tween 80 on glyconeogenesis in washed cell suspensions

As stated before, many strains of *Tetrahymena pyriformis* can convert fats into glycogen via the glyoxylate cycle. The ability of a lipid substrate to stimulate glyconeogenesis would be an indication that the glyoxylate cycle operates in the system described. Acetate, however, was non-stimulatory in strain GL. For this reason three other lipid substrates were tested for glycogen production: Tween 40 (polyoxyethylene sorbitan monopalmitate), Tween 80 (polyoxyethylene sorbitam monooleate) and Lecithin. Tween 40 and Tween 80 were hoped to be good substrates because Kidder et al. (50) showed that the presence of Tween 80 in the growth medium was nontoxic to Tetrahymena cells and that during growth a large amount of lipid accumulated inside the cells. Lecithin was used as a substrate because Wagner (2) showed that, during the enodgenous glyconeogenesis the lipids that are depleted in the cell are the phospholipids. The amount of phospholipid consumed was sufficient to account for the glycogen synthesized during the incubation time.

All the substrates were added at final concentration of 0.1%. Table 8 summarizes the results of three experiments testing Tween 40

TABLE 8

Stimulatory Effect of Tween 40 and Lack of Effect of Tween 80 on Glyconeogenesis[a,b]

Substrate Added	Experiment #1	Experiment #2			Experiment #3
	Factor of Stimulation	μmoles Glycogen (Glucose) Above 0 Time	Factor of Stimulation	μmoles NH_3 Above 0 Time	Factor of Stimulation
NONE	1.00	1.18	1.00	4.10	1.00
L-proline (1mM)	–	1.43	1.21	4.57	2.06
L-proline (2mM)	2.13	1.76	1.49	5.32	-
Tween 40	1.69	1.49	1.26	3.82	1.29
Tween 80	1.06	-	-	-	-
proline (2mM) + Tween 40	2.17	-	-	-	-
proline (2mM) + Tween 80	2.09	-	-	-	-
proline (1mM) + Tween 40	-	1.83	1.55	5.00	-

a,b as in legend, Table 1, p. 35.

and Tween 80 as substrates for glyconeogenesis. From the results of experiment #1 it can be concluded that Tween 80 is a poor substrate for glyconeogenesis. In contrast, Tween 40 stimulated glyconeogenesis 1.7-fold over the endogenous increase. In Experiment #2 (Table 8) the mixture of proline and Tween 40 was tested with proline in limiting concentration (1 mM). In this experiment Tween 40 alone stimulated glyconeogenesis to the same extent as 1 mM proline (1.26-fold). Addition of the mixture of Tween 40 and 1 mM proline improved glyconeogenesis significantly (1.55-fold stimulation). The glycogen content of the cells after 4 hours of incubation in this case is slightly higher than the glycogen produced with a mM proline.

Parallel to the determination of the glycogen content of the cells, in the same experiment (Exp. #2, Table 8), the production of ammonia was measured. Addition of proline to the incubation medium stimulated ammonia production, and the ammonia produced was dose dependent. Tween 40 had no marked effect on ammonia production--the ammonia produced was only slightly lower than the endogenous increase. Thus, even though Tween 40 is glycogenic, the endogenous glyconeogenesis does not seem to be significantly affected. This is contrary to the work of Wagner (2), who reported that addition of acetate to washed cell suspensions (strain E) abolished endogenous ammonia production. When Tween 40 and proline (1 mM) are incubated with cells for 4 hours, a significant raise in glycogen level is accompanied by a release of ammonia that appears to be significantly higher than in the case of 1 mM proline only. These results indicate that Tween 40 improves the utilization of proline and/or endogenous amino acids by the cells.

Figure 5: Lack of effect of lecithin on glyconeogenesis in washed cell suspensions.

Cells were harvested from 41 hours old GL/PPLE cultures.

Designation:

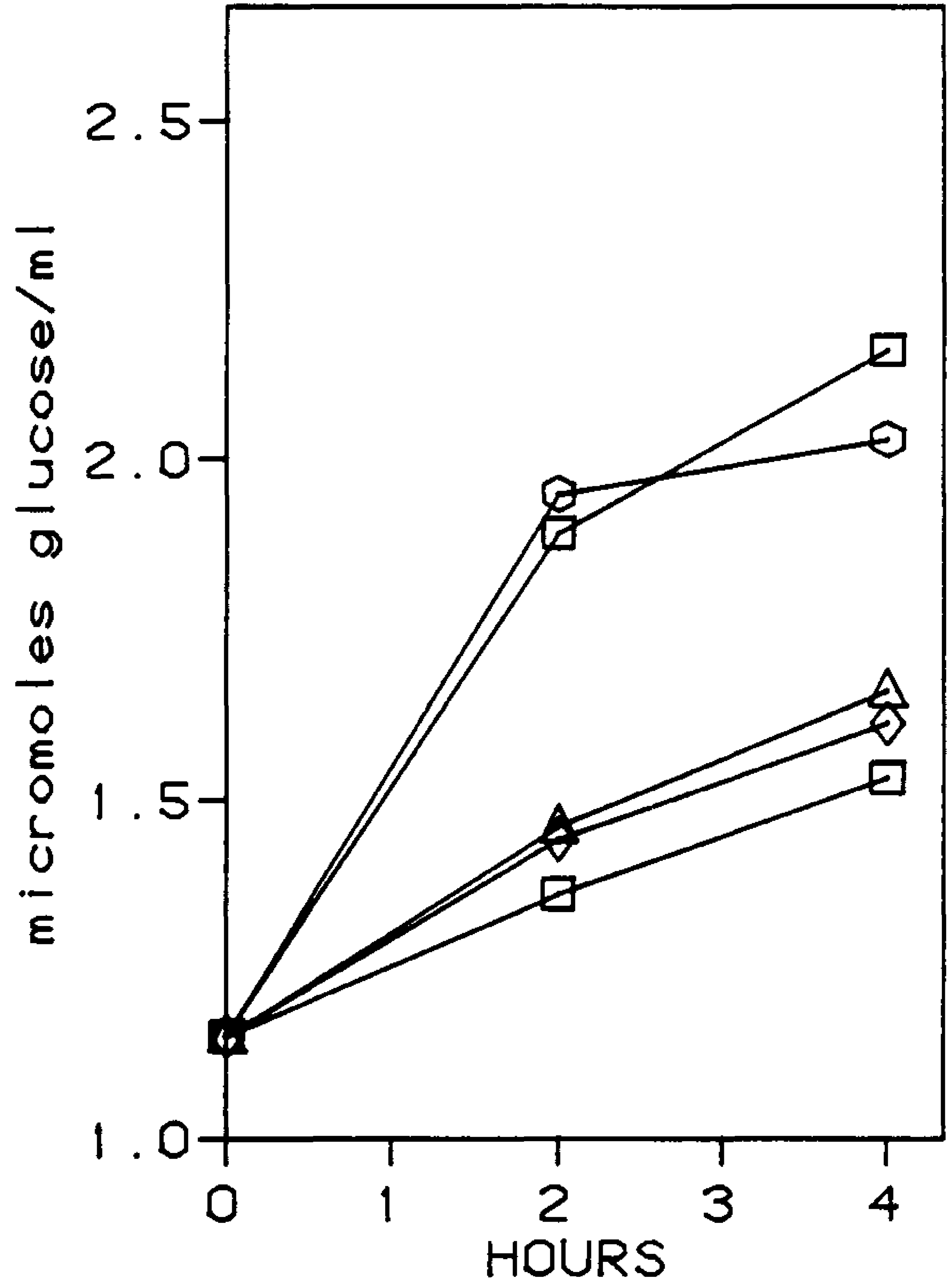
Diamonds - Endogenous incubation

Triangles - Na acetate (1 mg/ml)

Hexagons - L-proline (2 mM)

Squares - Lecithin (0.1%): lower red curve
Lecithin (0.1%) + L-proline (2mM): upper red curve.

Figure 5



An experiment showing the lack of effect of lecithin on glyconeogenesis is shown in Figure 5. It can be seen in Figure 5 that after 2 hours of incubation, there was no increase in glycogen level upon addition of lecithin or acetate, whereas in the incubations containing L-proline (2 mM), the glycogen synthesis was greatly stimulated. A mixture of lecithin and proline stimulated glyconeogenesis to the same extent as proline (2 mM) alone. Acetate did not stimulate glyconeogenesis in this experiment (Figure 5).

In summary, among the lipid substrates tested, only Tween 40 appeared to stimulate glyconeogenesis in washed cell suspensions. Addition of proline in limiting amount (1 mM) in presence of Tween 40, enhanced significantly both glyconeogenesis and ammonia production of the cells, as compared to the appropriate controls. Tween 80, lecithin and acetate had no effect on glyconeogenesis.

F. Metabolic fate of tracer acetate and tracer proline

The incorporation of 2-^{14}C-acetate and U-^{14}C-L-proline into major cellular fractions and CO_2 was studied in order to confirm by means of tracers the results obtained in balance studies. The results are summarized in Table 9.

On the basis of the balance studies, the % efficiency of the conversion of the carbon atoms of L-proline into glycogen was calculated to be about 35% (See Table 3). However, only 7.3% of the total tracer was incorporated into glycogen fraction. In contrast, the incorporation of ^{14}C from 2-^{14}C-acetate exceeds the expectation based upon the lack of stimulation of glyconeogenesis by acetate. The tracer experiment thus did not support the balance study and, therefore, an

TABLE 9

Metabolic Transformation of 2-^{14}C-acetate and U-^{14}C-proline (sample experiment).

Washed cells, obtained from 40 hours old GL/PPLE cultures, were incubated for 4 hours as described in "Experimental Procedures".

0.25 microcuries of tracer was added to each incubation.

Na Acetate and L-proline was added at 5 microatoms (C)/ml.

TABLE 9

Substrate	CO_2	GLYCOGEN	PROTEIN	LIPID	NUCLEIC ACIDS	ACID SOLUBLE FRACTION
2-[^{14}C]-acetate + Na Acetate	47.2%	10.1%	6.7%	6.7%	0.8%	28.4%
U-[^{14}C]-proline + L-proline	16.7%	7.3%	4.2%	2.1%	0.7%	69.0%

experiment was designed to study the fate of L-proline in detail in order to explain this discrepancy. The results are reported in the next section (Section G).

In case of tracer acetate the pattern of incorporation varied from experiment to experiment. In the experiment described a large fraction of the tracer was converted to CO_2 (47.2%). The incorporation into the glycogen fraction was 10.1%. Since the balance studies showed no stimulation of glyconeogenesis by acetate, this figure is higher than expected. However, the large amount of oxidation of acetate by the cells provides much opportunity for non-specific labeling by exchange with metabolic intermediates. The results demonstrate that the cells can utilize acetate, but they do not use it to make net glycogen. Instead they use it as fuel for respiration or for lipid synthesis (in one experiment about 30% of ^{14}C from 2-^{14}C-acetate was incorporated into the lipid fraction).

G. Metabolism of L-proline in *Tetrahymena pyriformis*, GL

Preliminary experiments indicated that the incorporation of radioactive label from U-^{14}C-L-proline into glycogen (Table 9) in washed cell suspensions was much lower than the one predicted from the balance studies (Table 1). Thus, in order to clarify the fate of L-proline under glyconeogenic conditions, a combined experiment was designed to measure these factors under like conditions:

1) Endogenous glyconeogenesis.
2) Proline-stimulated glyconeogenesis.
3) NH_3 production in (1).
4) NH_3 production in (2).

5) ^{14}C-proline uptake from the medium in (2).

6) Distribution of ^{14}C-proline incorporated into the cells among the 71% alcoholic extract, glycogen, CO_2, protein, nucleic acids and lipids.

7) Determination of the content of radioactive proline and its small metabolites in the incubation medium and in the 71% alcoholic extract of the cells by chromatography.

8) Determination of the content of radioactive proline and its amino acid metabolites in the protein hydrolysate of the cells by chromatography.

The various techniques utilized are described in "Experimental Procedures".

1. Proline-stimulated glyconeogenesis and ammonia production

Figure 6 represents glyconeogenesis and NH_3 production with and without L-proline added to the cell suspension. As can be seen, after 2 hours incubation, addition of L-proline caused synthesis of an extra 0.75 micromoles glucose/ml. At the same time addition of L-proline stimulated greatly NH_3 production. After 2 hours, an extra 1.6 micromoles of ammonia/ml was produced in the presence of proline. After 4 hours the differences became smaller; therefore, for quantitative comparisons, I will refer to the state of affairs after 2 hours only.

The concentration of proline in this experiment was 2 micromoles/ml. If all of the extra ammonia produced was due to L-proline catabolism, it would signify 80% utilization of proline (1.6/2 x 100%). Since from one molecule of proline we may obtain in a

Figure 6: Effect of L-proline on glyconeogenesis and ammonia production of washed cell suspensions.

The conditions of the incubations are described in the text. At time 0 the cells contained 0.75 micromoles glycogen/ml and 3.35 micromoles of ammonia/ml.

Designation:

Diamonds

a. black - glycogen (endogenous)

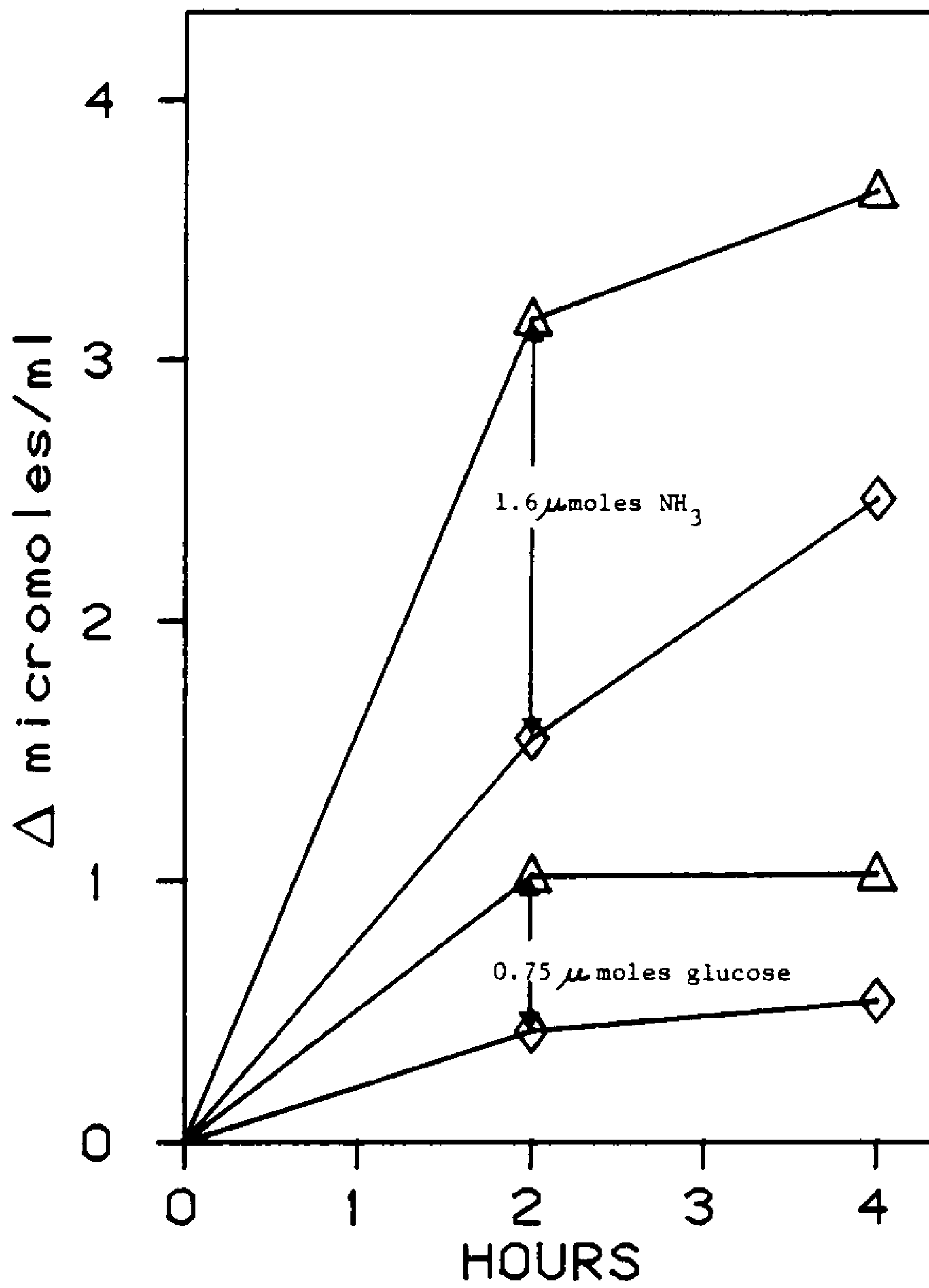
b. red - ammonia N (endogenous)

Triangles

a. black - glycogen with L-proline (2 mM)

b. red - ammonia N with L-proline (2 mM)

Figure 6



series of metabolic reactions one molecule of PEP, two molecules of proline are needed to make one glucose molecule. Therefore the stoichiometric relationship between L-proline and glucose is:

2 micromoles proline 1 micromole glucose

Thus from 1.6 micromoles L-proline, a maximum of 0.8 micromoles glucose could be made. In reality we find 0.75 micromoles of extra glucose produced in the presence of proline, which is 94% of the calculated value.

2. Uptake of U-^{14}C-L-proline from the medium

Uptake of ^{14}C-proline into the protozoan cells is shown in Figure 7. As can be seen, the cell content of radioactivity rose steadily for 2 hours, until it reached about 60% of the total radioactivity. For the remaining time of the incubation a steady-state is established and the intracellular radioactivity level is maintained at about 60% of the total.

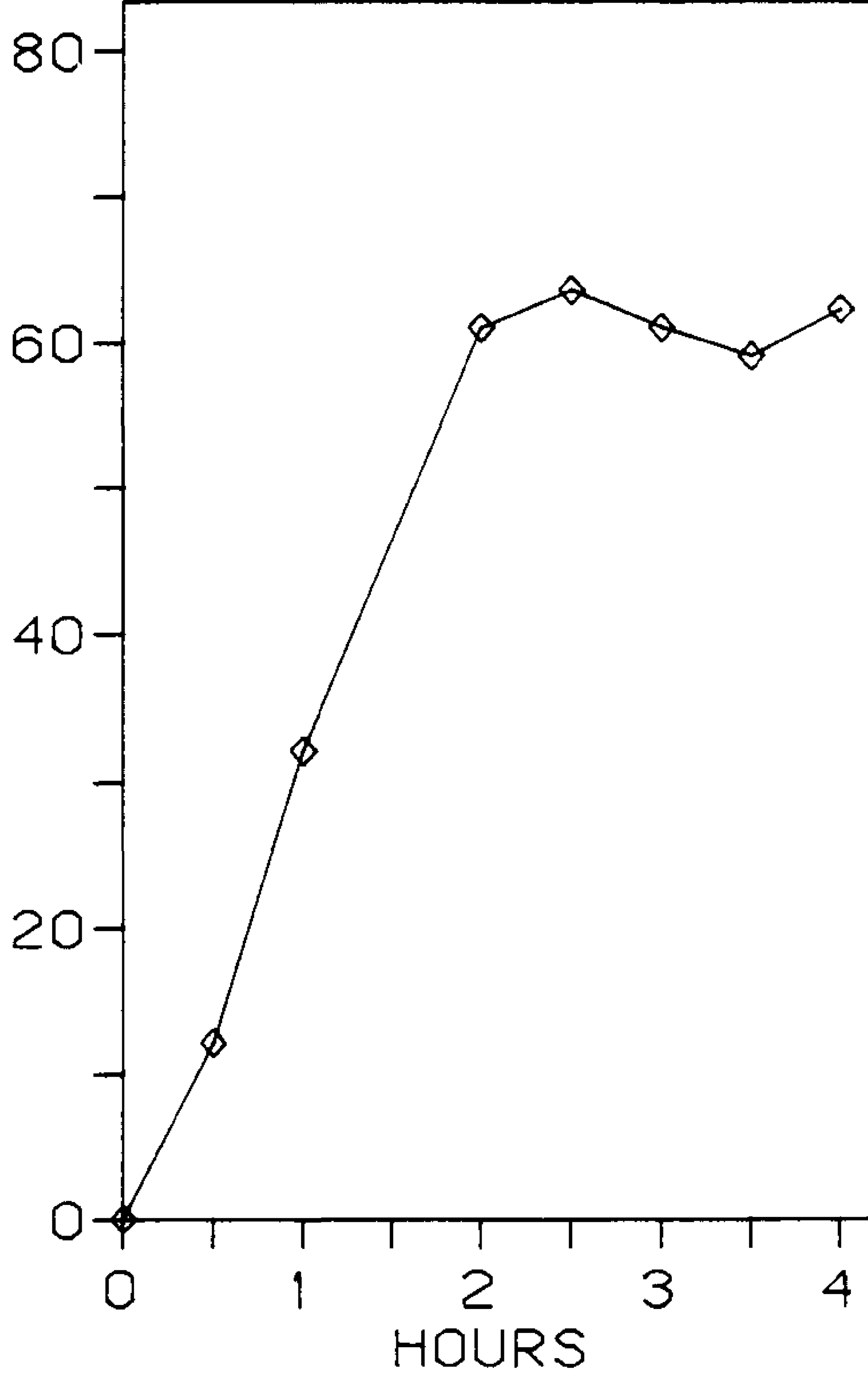
3. Incorporation of ^{14}C from U-^{14}C-L-proline into CO_2, glycogen, protein, lipid, nucleic acids and alcohol-soluble fraction

Before we proceed with the discussion of the experimental results, it would be useful to calculate the percent incorporation of ^{14}C from radioactive proline into glycogen and CO_2 fractions, predicted on the basis of the net glyconeogenesis and ammonia production experiment (this section, part 1). This can be then compared with the percentages actually found in the experiment.

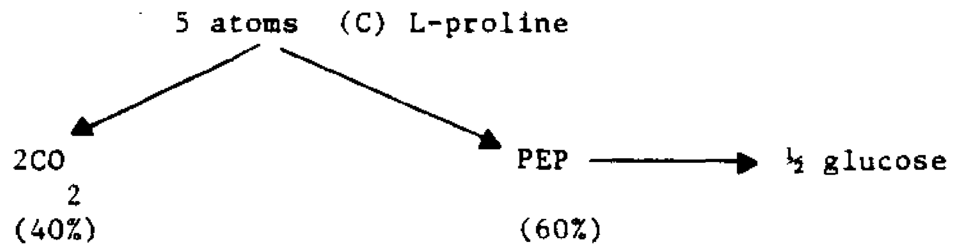
Figure 7: Uptake of U-^{14}C-L-Proline by the cells.

Washed cell suspensions were incubated with 2.0 mM L-proline containing 0.25 microcuries of U-^{14}C-L-proline. Loss of radioactivity from suspension medium was determined at indicated times as described in "Experimental Procedures".

Figure 7



The fate of the carbons of L-proline is depicted below:



As shown, the maximum incorporation of ^{14}C from U-^{14}C-L-proline into glycogen can be 60%. However, the data for ammonia production indicated that only 80% of L-proline was utilized, and therefore the predicted % of radioactivity incorporated into glycogen should be 60% x .8 = 48%. In addition, the glycogen data suggested that only 94% of the maximum possible glucose was produced. Thus the maximum predicted value of ^{14}C incorporation into the glycogen fraction becomes 48% x 0.94 = 45%. On the basis of the diagram shown, the ratio of %^{14}C in glycogen/ %^{14}C in CO_2 should be 3/2. Therefore the predicted value for fraction of the radioactivity in the CO_2 fraction is 45% x 2/3 = 30%.

Table 10 summarizes the distribution of ^{14}C from U-^{14}C-L-proline among the major fractions of the cells.

The distribution of radioactivity after 2 hours of incubation is essentially the same as after 4 hours; therefore I will concentrate on the "2 hours" results.

The chemical balance study predicted 80% utilization of L-proline. However, tracer data show that 42% of L-proline remained unused after 2 hours of incubation. The balance study predicted that 45% of the total ^{14}C should be incorporated into the glycogen fraction. However, the tracer incorporation into glycogen was found to be only 12.4%.

TABLE 10

Incorporation of ^{14}C from U-^{14}C-L-proline into Major Cellular Fractions[a]

TIME	% INCORPORATION[b] OF ^{14}C INTO									
	CO_2	GLYCOGEN	PROTEIN	LIPID	NUCLEIC ACIDS	71% ALCOHOLIC EXTRACT OF CELLS		INCUBATION MEDIUM		TOTAL
2 hrs	18.1%	12.4%	3.0%	0.6%	0.4%	24.8%		40.7%		100%
						Proline	Others	Proline	Others	Total Unused Proline
						20.5%	4.3%	22.9%	17.8%	43.4%
4 hrs	18.5%	14.2%	3.3%	1.1%	1.0%	20.8%		41.1%		100%
						Proline	Others	Proline	Others	Total Unused Proline
						17.2%	3.6%	23.2%	17.9%	40.4%

a. Legend Table 1, proline 2 mM + 0.125 microcuries U-^{14}C-L-pro/ml.

b. Calculated as described in "Materials and Methods."

This is less than one-third of the expected value. Where is the missing radioactivity to be found?

The predicted value for the tracer content of CO_2 is 30%. The experimental value is 18.1%. The incorporation into protein was very low (3.0%), and there was negligible incorporation of ^{14}C into lipid and nucleic acid fractions.

The supernatant incubation medium contains 40.7% of the total ^{14}C from proline after 2 hours. Upon chromatography it could be estimated that the cell supernatant contained 22.9% of the total ^{14}C in the form of unused proline and the remainder contained 17.8% of the total ^{14}C in other amino acids (Figure 8). The "71% alcoholic extract of the cells" after 2 hours contains 24.8% of the total administered radioactivity. It contains 20.5% of the total ^{14}C in the form of unused L-proline (Figure 9) and only 4.3% of the total tracer as other amino acids. Thus it seems reasonable to conclude that much of the L-proline is metabolised to amino acids which for the most part are excreted into the incubation medium as soon as they are formed.

The sum of the percentages of ^{14}C content in amino acids other than proline is 22.1% of the total ^{14}C. This may in part answer the question on the radioactivity presumably missing from the glycogen fraction.

In summary, there are contradictions between balance studies and tracer studies of L-proline metabolism in the ciliate:

1) The NH_3 production suggests 80% utilization of L-proline after 2 hours. The tracer experiment shows only 60% utilization (40% of the L-proline is left unused).

2) It is predicted from balance studies that 45% of the tracer in U-^{14}C-proline should be incorporated into glycogen.

Figure 8: Proline and its metabolites in cell supernatant (2 hours).

Paper chromatography was performed as described in "Experimental Procedures". The relative content of radioactivity in the chromatograms was estimated by cutting out the peak and weighing it on analytical balance.

Components of the cell supernatant

	% of total radioactivity
Peak #1 - L-proline	56.3%
Others -	43.7%

Figure 8

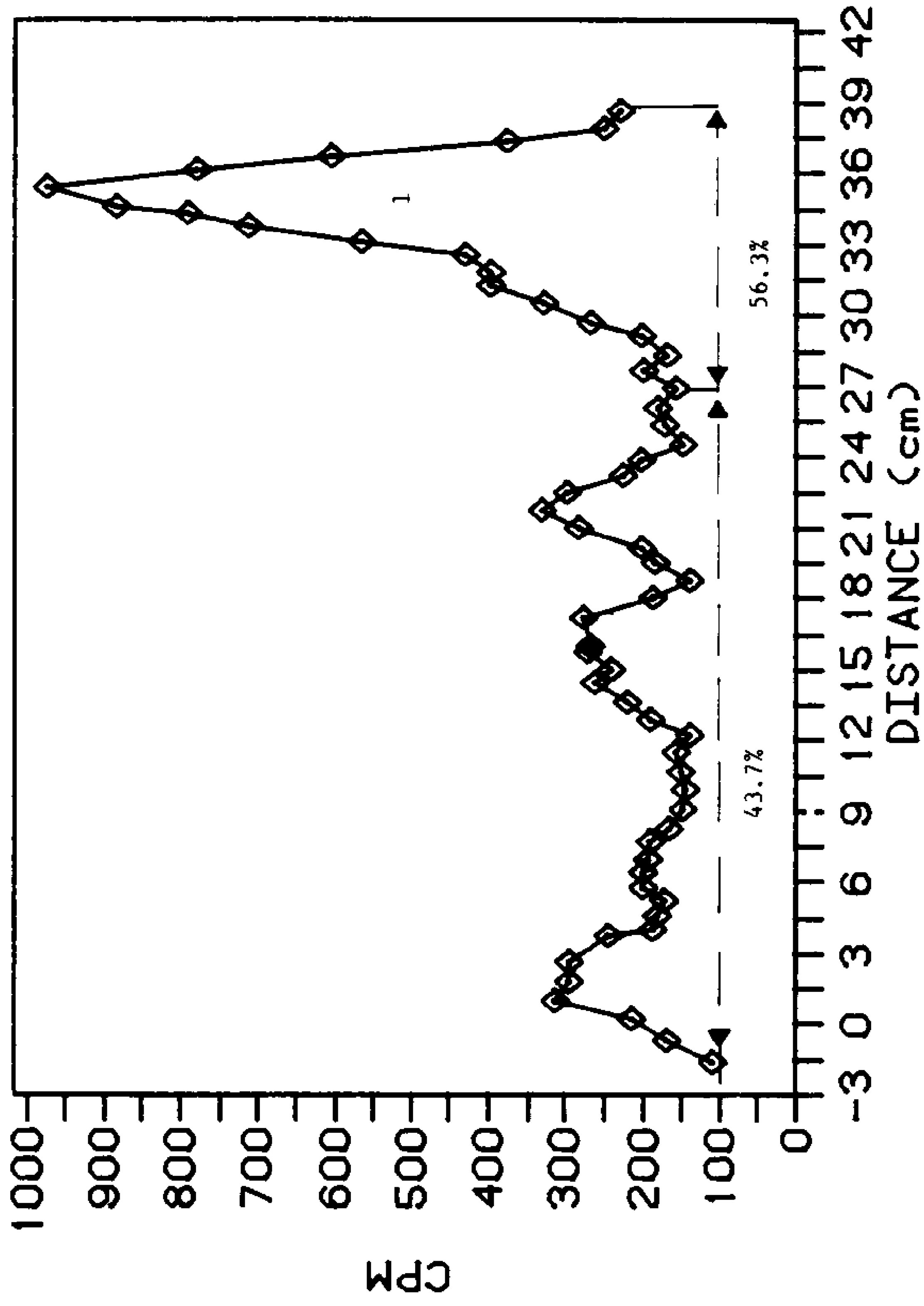


Figure 9: Proline and its metabolites in the alcohol extract of the cells after 2 hours of incubation.

Alcohol extract of the cells was obtained and chromatographed as described in "Experimental Procedures".

The relative content of the radioactivity in the chromatograms was estimated by cutting out the peak and weighing it on analytical balance.

Components of the alcohol extract:

	% of total radioactivity
Peak #1 (proline)	82.5%
Others	17.5%

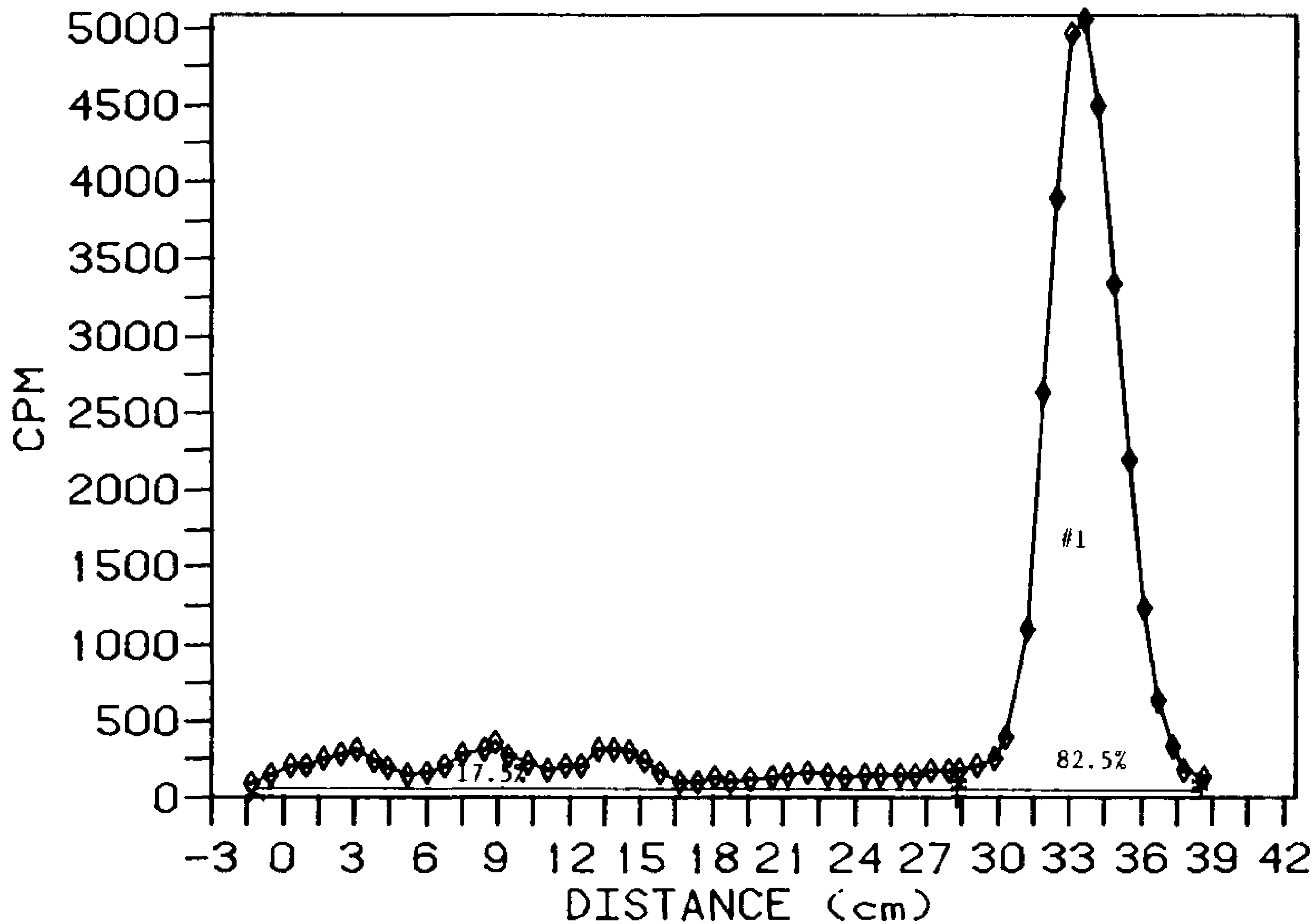


Figure 9

However, only 12% of the total ^{14}C is found in the fraction.

3) A large portion (21.5%) of the total radioactivity appears in the form of amino acids other than proline. This means that the amount which could produce ammonia was only 38.5%, thus making the gap between the NH_3 balance studies and the tracer studies even wider.

These results suggests two occurrences:

1) Not all of the extra ammonia produced derives from the added proline; thus an additional (endogenous) source of NH_3 must be present.

2) The glycogen formed due to the presence of L-proline is only in minor part synthesized from the carbon atoms of L-proline, thus another substrate for glyconeogenesis must be present.

The key to the mechanism of additional glyconeogenesis caused by L-proline seems to reside in the transformation of L-proline to other amino acids. We shall proceed therefore by looking at the amino acid metabolites of L-proline.

4. <u>Amino acid metabolites of L-proline in Tetrahymena pyriformis</u>

The amino acid metabolites of L-proline in Tetrahymena pyriformis were sought by paper chromatography of the appropriate fractions. The results are summarized in Table 11. Three fractions were analysed: the 71% alcoholic extract of the cells, the cell supernatant and the cellular protein fraction (after hydrolysis). The results are discussed below.

Figure 10: Metabolites of proline in alcohol extract of the cells (2 hours) - expansion of lower two-thirds of Figure 9.

Designation:

		17.3% (Total)
A.	aspartate -	5.2%
B.	glutamate -	7.3%
C.	asparagine -	5.2%

The black line represents the expansion of the lower two-thirds of Figure 9. The red line represents chromatogram of alcohol extract acid hydrolysate. Hydrolysis was done in the same way as described in "Experimental Procedures" under "Protein Hydrolysate".

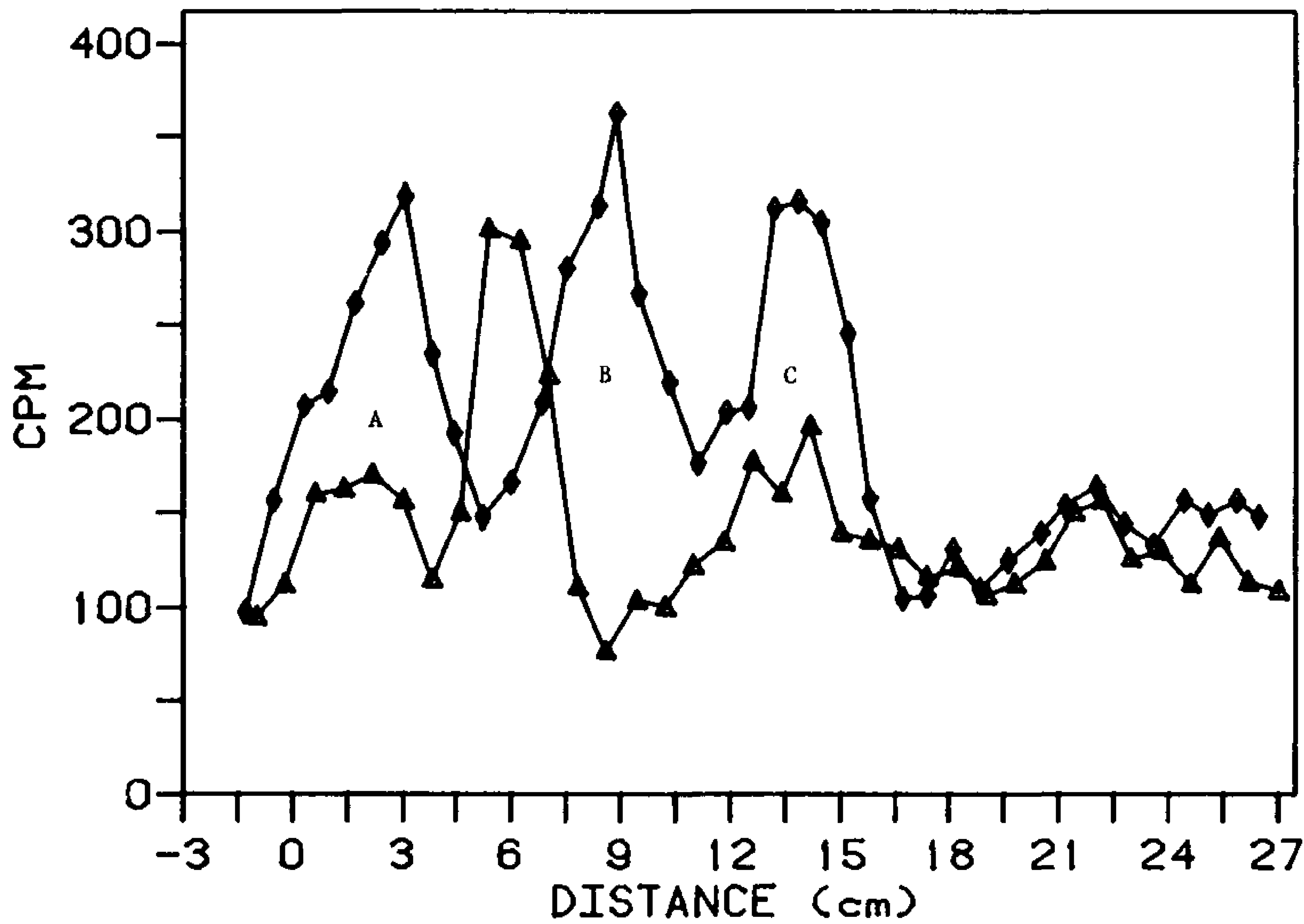


Figure 10

4.1 Amino acid composition of the 71% alcoholic extract of the cells.

After 2 hours of incubation with L-proline (2 mM) with added tracer proline, the alcoholic extract was obtained as described in "Experimental Procedures". The total radioactivity found in the extract was 24.8%. Paper chromatography showed a major peak corresponding to proline that contained 82.7% of the radioactivity applied to the chromatogram. The remaining radioactivity (17.3%) was located in three minor peaks (Figure 10). The first two peaks were identified as aspartate and glutamate and contained 5.2% and 7.3% of the radio-activity respectively. The third peak chromatographed with L-asparagine but it had also an R_f very similar to glycine. In order to differentiate between the two amino acids, the alcoholic extract was subjected to acid hydrolysis. Asparagine would be hydrolyzed in the treatment. Then the extract was rechromatographed. As can be seen (red line, Figure 10), the peak corresponding to asparagine diminished significantly. Thus asparagine appears to be the major component of this peak, which contained 5.2% of the radioactivity applied to the chromatogram.

4.2 Amino acid composition of the cell supernatant of the cells after incubation.

After 2 hours of incubation of the cells with 2 mM proline with added tracer proline, the cell supernatant contained 40.7% of the total radioactivity. Paper chromatograms of this fraction contained 5 radioactive peaks. A major peak that corresponded to proline contained 56.3% of the radioactivity applied to the chromatogram (Figure 8,

Figure 11: Metabolites of L-proline in the cell supernatant (2 hours) -- expansion of the lower two-thirds of Figure 8.

The black line represents the expansion of the lower two-thirds of Figure 8. The red line represents chromatogram of cell supernatant acid hydrolysate.

The peaks were identified as:

			% of radioactivity
A.	aspartate	(#1)	12.2%
B.	glutamate	(#2)	5.1%
C.	asparagine	(#3)	12.9%
D.	glutamine	(#4)	13.5%

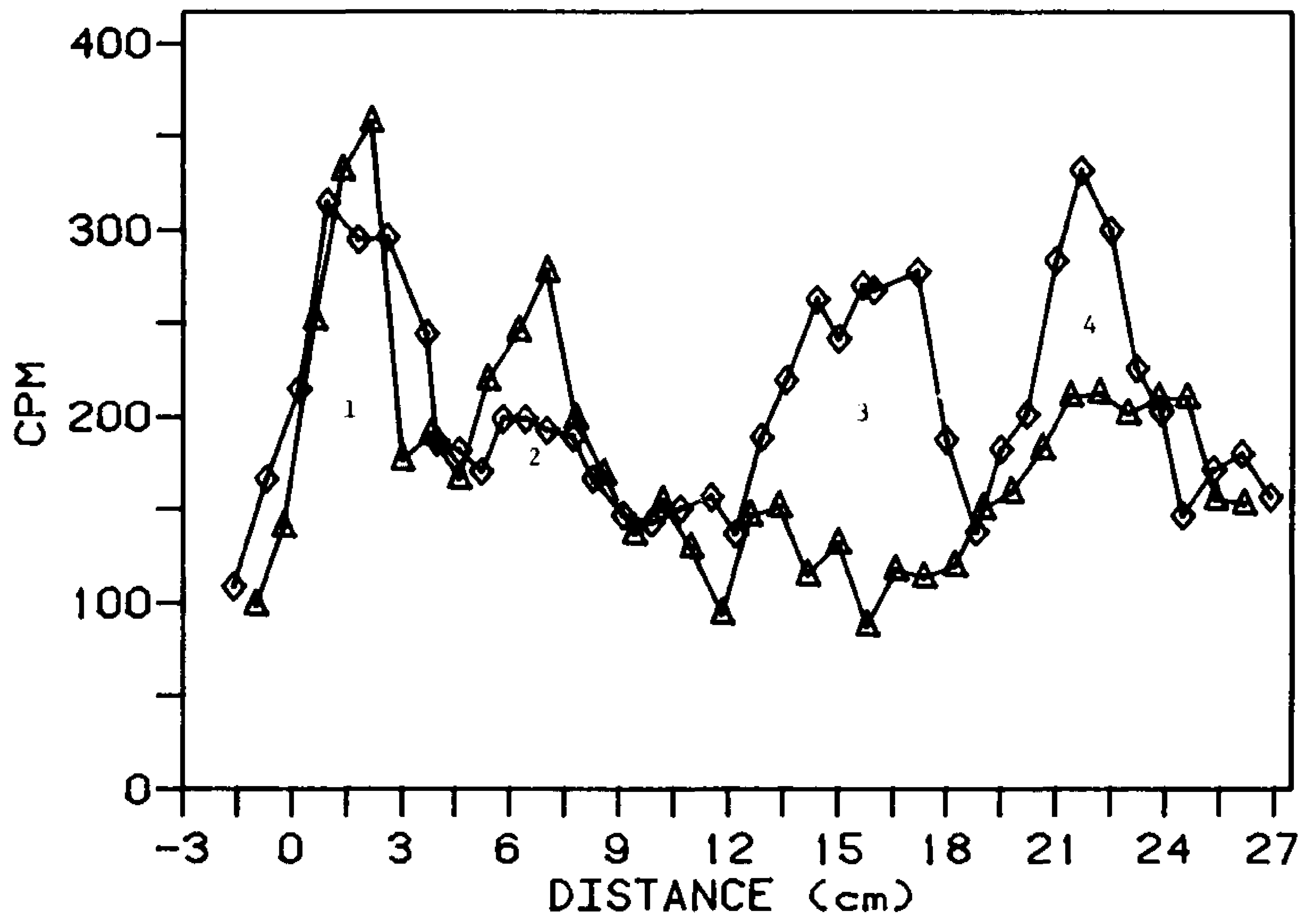


Figure 11

Table 10). The other peaks corresponded to aspartate (peak #1), that contained 12.2% of radioactivity, and glutamate (peak #2), containing 5.1%. Peak #3 cochromatographed with L-asparagine; however glycine had a very similar R_f. Peak #4 cochromatographed with L-glutamine, but L-alanine had a very similar R_f. In order to exclude one of the possibilities, the supernatant was hydrolyzed in acid. Such treatment would hydrolyze asparagine and glutamine and, therefore, the peaks would be expected to disappear. The hydrolyzed supernatant was chromatographed (1 red line, Figure 11). It can be seen that upon acid hydrolysis the content of peaks 3 and 4 were reduced remarkably, suggesting that the major components of those peaks were asparagine and glutamine. Some alanine, however, may be present (peak 4, Figure 11). Thus asparagine in the cell supernatant contained 12.9% of the radioactivity applied to chromatogram and glutamine, 13.5%.

4.3 Amino acid composition of the protein hydrolysate of the cells.

When amino acids are produced from L-proline, they can be incorporated into the protein fraction. Therefore cells were incubated with L-proline (2 mM), containing 2 microcuries of the tracer proline. Subsequently a protein hydrolysate was prepared as described in "Experimental Procedures".

By means of paper chromatography three radioactive peaks were resolved and identified by cochromatography: L-proline, L-glutamate and L-aspartate (Figure 12). The proline peak contained 50% of the radioactivity applied to the chromatogram, glutamate 31% and aspartate 15% (Table 11).

Figure 12: Metabolites of proline-incorporation into protein.

Protein hydrolysate was prepared as described in "Experimental Procedures".

The red line represents the paper chromatogram of protein hydrolysate. In order to resolve the lower peak, the chromatogram was dried and developed once more. Three peaks were identified in this way:

		% of radioactivity
A.	aspartate	19%
B.	glutamate	31%
C.	proline	50%

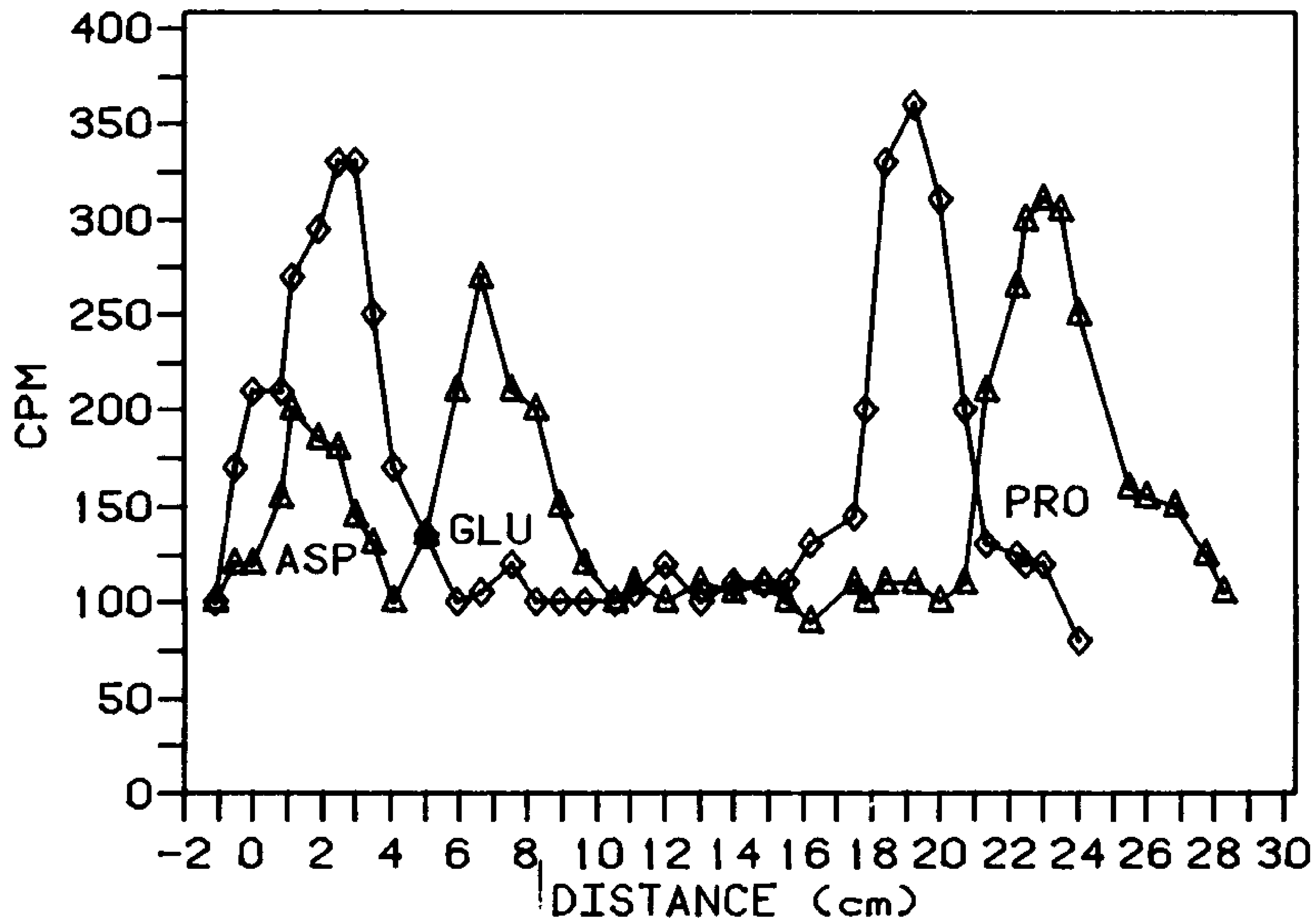


Figure 12

TABLE 11

Amino Acid Metabolites of L-proline

FRACTION	Amino acids found	Fraction of radioactivity applied to chromatogram %	Incorporation of ^{14}C into Fraction %	Fraction of total radioactivity supplied to cells in each amino acid %
Cells: A-alcohol extract (2 hours)	L-proline	82.7%	24.8%	20.5%
	L-glutamate	7.3		1.8
	L-aspartate	5.2		1.3
	L-asparagine	5.2		1.3
B-protein hydrolysate	L-proline	50%	3%	1.5%
	L-glutamate	31		0.9
	L-aspartate	19		0.6
Incubation Medium (2 hours)	L-proline	56.3%	40.7%	22.9%
	L-glutamate	5.1		2.1
	L-aspartate	12.2		5.0
	L-asparagine	12.9		5.2
	L-glutamine	13.5		5.5

Table 11 summarizes the results obtained by paper chromatography. These results indicate that proline is metabolized to glutamate in Tetrahymena pyriformis. The other amino acids detected can be obtained form glutamate. This is in agreement with Dewey et al (51) who found that when tracer proline was added to cultures of Tetrahymena pyriformis, strain W, it was utilized well by the cells with the end products being CO_2 and glutamate. In contrast to the results of Dewey et al (51), forms of aspartate, not glutamate were the major amino acid components, other than proline, of the cell supernatant in our experiments.

More than 80% of the radioactivity in the 71% alcoholic extract (which represents the intracellular amino acid pool) was found to be nonmetabolized proline and about 20% was found to be in other amino acids. Glutamate was detected in highest amount but aspartate and asparagine were also detected. It is of interest to point out that glutamine could not be detected inside the cells. The cell supernatant presents a different picture: only 56% of the radioactivity found in this fraction was associated with unused proline and the rest of the radioactivity was found to be associated with other amino acids: glutamine, asparagine, aspartate and glutamate in that order for ^{14}C content. This indicates that amino acids formed inside the cells from proline are rapidly excreted from the cells into the medium. It is interesting to note the absence of intracellular glutamine and the presence of a large amount of extracellular glutamine after 2 hours.

H. Metabolism of L-leucine in *Tetrahymena pyriformis*

As shown earlier (Section C, Table 3) L-leucine stimulated both glyconeogenesis and ammonia production under the conditions employed in these experiments. L-leucine is the only purely ketogenic amino acid and thus in the presence of glyoxylate cycle it should be converted primarily to glycogen. In order to learn the fate of leucine in the protozoan cell, an experiment was designed to study parallelly:

1) glyconeogenesis with and without leucine
2) ammonia production with and without leucine
3) incorporation of tracer leucine into major cellular fractions
4) uptake of leucine from the medium during the incubation time.

1. Leucine-stimulated glyconeogenesis and ammonia production

Figure 13 represents glyconeogenesis and NH_3 production with and without leucine added to the cell suspension. After 2 hours of incubation, added leucine caused an extra 0.33 micromoles glucose/ml. At the same time ammonia production was greatly stimulated by leucine--an extra 1.21 micromoles of ammonia was produced in the presence of leucine.

The concentration of leucine in this experiment was 2 mM. If all of the extra ammonia came from leucine, there was 60.5% utilization of leucine (1.21/2 x 100%).

The metabolic fate of leucine is depicted in Appendix, diagram 1. From 1 mole of leucine, 3 moles of Acetyl CoA can be obtained. Assuming that all three molecules enter glyoxylate cycle, they would

Figure 13: Effect of L-leucine on glyconeogenesis and ammonia production of washed cell suspensions.

Cells were obtained from 41 hours old PPLE cultures. 2% (v/v) cell suspensions in RRP were incubated with and without 2.0 mM L-leucine.

At time 0, the cells contained 0.22 micromoles glycogen/ml and 2.84 micromoles of amm/ml.

Designation:

Diamonds

a) black - glycogen (endogenous)

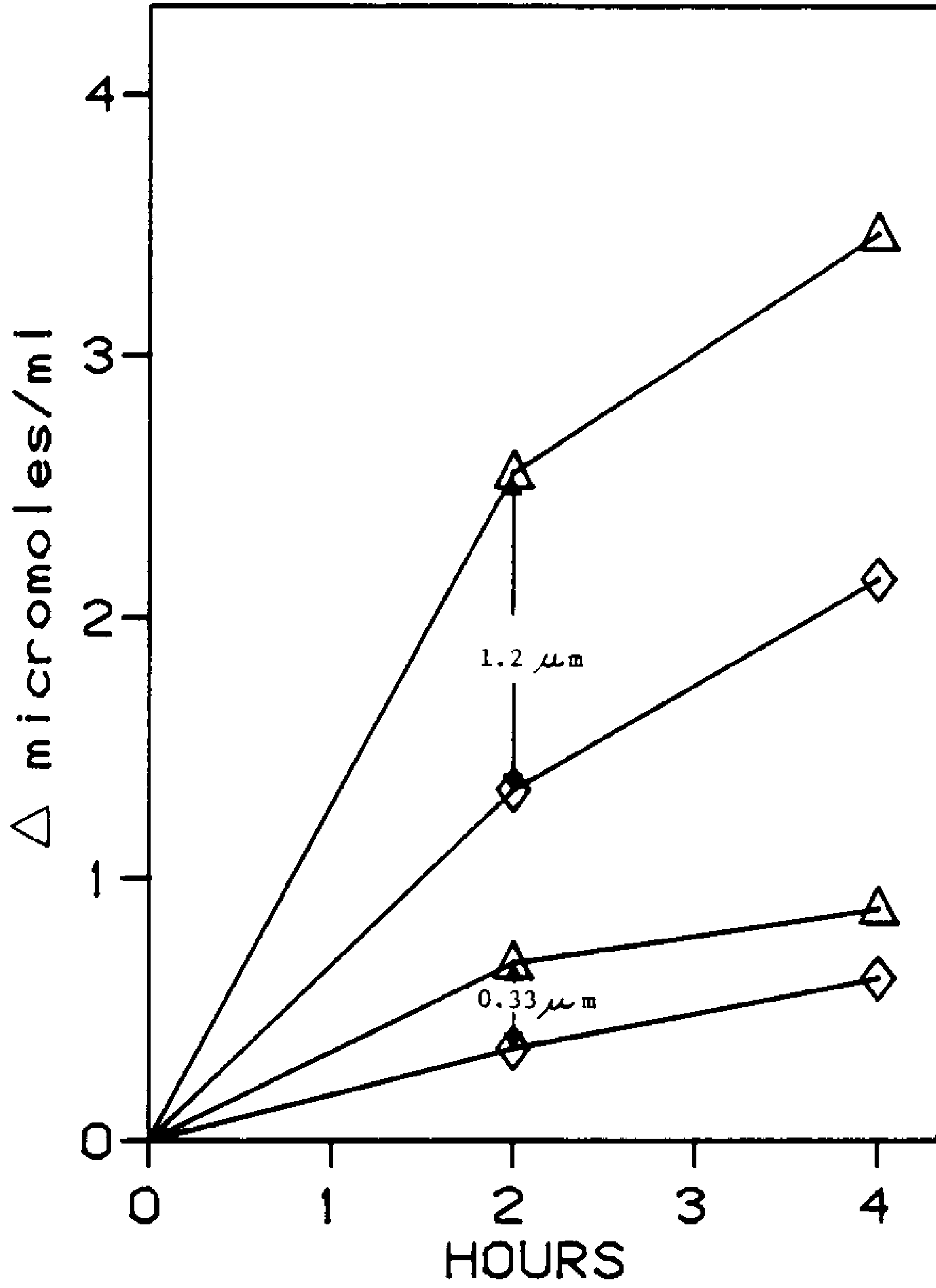
b) red - ammonia N (endogenous)

Triangles

a) black - glycogen with leucine

b) red - ammonia with Leucine

Figure 13



give rise to 0.75 moles of glucose (see Appendix, diagram 1). Thus, from 1.21 micromoles of leucine, a maximum of 0.91 micromoles of glucose could be made. This maximal estimate assumes that no Acetyl CoA enters TCA cycle to be oxidized or is incorporated into lipids and/or protein of the cell. In the experiment 0.33 micromoles of extra glucose were produced, which is 36% of the calculated value.

2. Incorporation of ^{14}C from leucine into major cellular components and CO_2

Cells of Tetrahymena pyriformis, GL, were incubated for 2 hours with 2 mM Leucine containing 0.125 microcuries of U-^{14}C-L-leucine/ml. The results obtained (Table 12), as in the case of proline, can be compared to the values predicated on the basis of the balance study (see this section, part 1).

The fate of the 6 carbon atoms of leucine is depicted in Appendix Diagram 1. The diagram is made under the assumption that all the acetyl-CoA molecules, originating from proline, enter the glyoxylate cycle and that there is no incorporation of ^{14}C from leucine into protein and lipid fraction. This is therefore a maximal estimate for the % incorporation of label into the glycogen fraction.

The fate of the carbons of L-leucine, upon these assumptions, is depicted below:

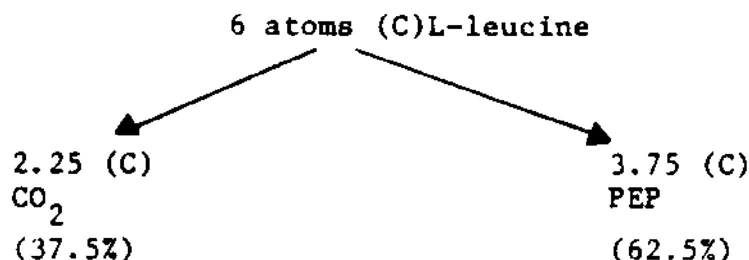


TABLE 12

Incorporation of ^{14}C from U-^{14}C-L-leucine[a] into Major Cellular Fractions

Substrate	% Incorporation of ^{14}C after 2 hours into[b]						
	CO_2	Glycogen	Protein	Lipid	Nucleic Acids	71% Alcoholic Extract of Cells	Cell Supernatant
U-^{14}C-L-leucine	14.4%	6.7%	12.4%	10.4%	2.0%	4.2%	50%
L-leucine (2mM) + U-^{14}C-L-leucine	18.8%	11.0%	7.3%	9.0%	1.8%	4.1%	48.5%

a - Legend, Table 1, p. 35.

b - Calculated as described in Materials and Methods.

As shown, the maximum incorporation of ^{14}C from radioactive leucine into glycogen can be 62.5%. However, the data for ammonia production indicated that only 60.5% of L-leucine was utilized by the cells. Thus the predicted % of radioactivity incorporated into glycogen becomes 37.8% (62.5% x .605). In addition the glycogen data suggested that only 36% of the maximal possible glycogen was produced. Therefore the predicted radioactivity content in glycogen fraction becomes 13.6% (37.8% x .36).

On the basis of the diagram showing the fate of the carbons of leucine, the ratio of % ^{14}C in glycogen/% ^{14}C in CO_2 should be 1.67. Thus the % ^{14}C in CO_2 fraction can be calculated to be 13.6%/1.67 = 8.2%.

Table 12 summarizes the distribution of ^{14}C from U-^{14}C-L-leucine among the major fractions of the cells. On the basis of the ammonia production, the utilization of leucine in this experiment was estimated to be about 60%. In the tracer experiment the ^{14}C content of the cell supernatant after 2 hours of incubation was 48.5% (51.5% utilization). On the basis of the glycogen production in the balance studies, the incorporation into the glycogen fraction was estimated to be 13.6%. In the comparable tracer experiment (with 2 mM leucine) this figure was 11%. Thus, within the experimental error the tracer experiment supports the results obtained in the balance studies.

The predicted value for the tracer content in the CO_2 fraction is 8.2%. The experimental value is 18.4%. This discrepancy can be explained by the oxidation of some of the acetyl CoA molecules obtained from leucine via the TCA cycle.

Incorporation into the lipid fraction accounted for 9% of the ^{14}C and into the protein fraction for 7.3%. Thus 16.3% of total ^{14}C supplied is not available for glycogen synthesis, in contrast with the assumption when the predicted values were calculated. The comparison between the incubations with leucine at tracer and substrate levels, reveals a shift in the cellular metabolism: in the presence of leucine at substrate level the % incorporation of tracer into CO_2 and glycogen goes up, whereas the incorporation of leucine into protein is significantly reduced. Thus, at the substrate levels of leucine cells appear to show increased glyconeogenesis.

These results of the balance studies are in a fairly good agreement with the results obtained using U-^{14}C-L-leucine as a probe of cell's metabolism. The experiments indicate that leucine stimulates glyconeogenesis in Tetrahymena pyriformis, GL, and that this stimulatory effect is mainly due to the ability of the ciliate to convert the carbon backbone of leucine into glycogen.

3. Stimulation of glyconeogenesis by a mixture of L-proline and L-leucine

An experiment was designed to study the effect of a mixture of proline and leucine on glyconeogenesis. Both amino acids were used at the level of 1 mM--a level that does not result in the maximal glyconeogenesis for either substrate alone. The results are summarized in Table 13.

As can be seen, the mixture containing proline and leucine is more stimulatory than either of the amino acids by itself. The effect of the mixture appears to be additive, i.e. the increment for the mix-

ture equals the sum of the single increments. This may indicate that leucine and proline enter the protozoan cells by separate transport sites and then are used for glyconeogenesis. Extra ammonia released in the case of the leucine/proline mixture is more than extra ammonia released in the presence of either amino acid by itself. It is also slightly higher than what would be expected from a simple additive effect. A mixture of proline and leucine appears to be a very potent one in respect of stimulation of glyconeogenesis by a concerted action of a glucogenic and ketogenic amino acid: the first one possibly stimulating the process occurring from the second one.

TABLE 13

Effect of Leucine/Proline Mixture on Glyconeogenesis and NH_3 Production [a]

SUBSTRATE (mM)	GLYCOGEN: Glycogen after 2 hours μm/ml	GLYCOGEN: Δ Glycogen above endogenous	GLYCOGEN: Stimulation Factor [b]	NH_3: NH_3 after 2 hours μm/ml	NH_3: NH_3 above endog-enous
1. None	0.35	-	1.00	1.34	-
2. Leucine (1 mM)	0.63	0.28	1.80	2.08	0.74
		Sum 0.65			Sum 1.68
3. Proline (1 mM)	0.72	0.37	2.06	2.28	0.94
4. Leucine (1 mM) + Proline (1 mM)	1.00	0.65	2.86	3.19	1.83

a, b, as in Legend, Table 1, p. 35.

DISCUSSION

In this work an attempt was made to characterize protein glyconeogenesis in Tetrahymena pyriformis. This organism possesses a glyoxylate cycle that enables it to convert fats into glycogen. In order to study the problem as defined above we established growth conditions that would produce cells of Tetrahymena pyriformis using proteins rather than fats in the glyconeogenic pathway.

The most potent stimulant among the single amino acids studied was found to be L-proline. Wagner (2), who contributed greatly to our knowledge of glyconeogenesis in the protozoa, did not test L-proline as a substrate for glyconeogenesis because it is not essential for growth and also shows a low intracellular content. Dewey et al. (51) studied proline metabolism in Tetrahymena pyriformis (strain W), although they did not address themselves to the issue of glyconeogenesis. They concluded that L-proline was efficiently utilized by the protozoan cells: growing cultures of the ciliate in synthetic medium were found to degrade radioactive proline to glutamate and CO_2. Under the conditions of their experiments no appreciable amount of proline carbon was converted to glycogen during the incubations.

As claimed by Dewey et al. (51), and as confirmed by us, proline is metabolized to glutamate in the organism. This pathway consists of two enzymatic reactions (52, 53). In the first reaction proline is converted to Δ^1-Pyrroline 5-carboxylate* by proline dehydrogenase (53).

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*Abbr.: P5C

R. Kramar (54) showed that this dehydrogenase reaction did not depend on pyridine nucleotides. The enzyme was shown to be a membrane-bound flavoprotein (55), that was inhibited by L-lactate, L-pyruvate and, to a smaller extent by D-lactate in E. Coli (55) and the mammalian liver (53).

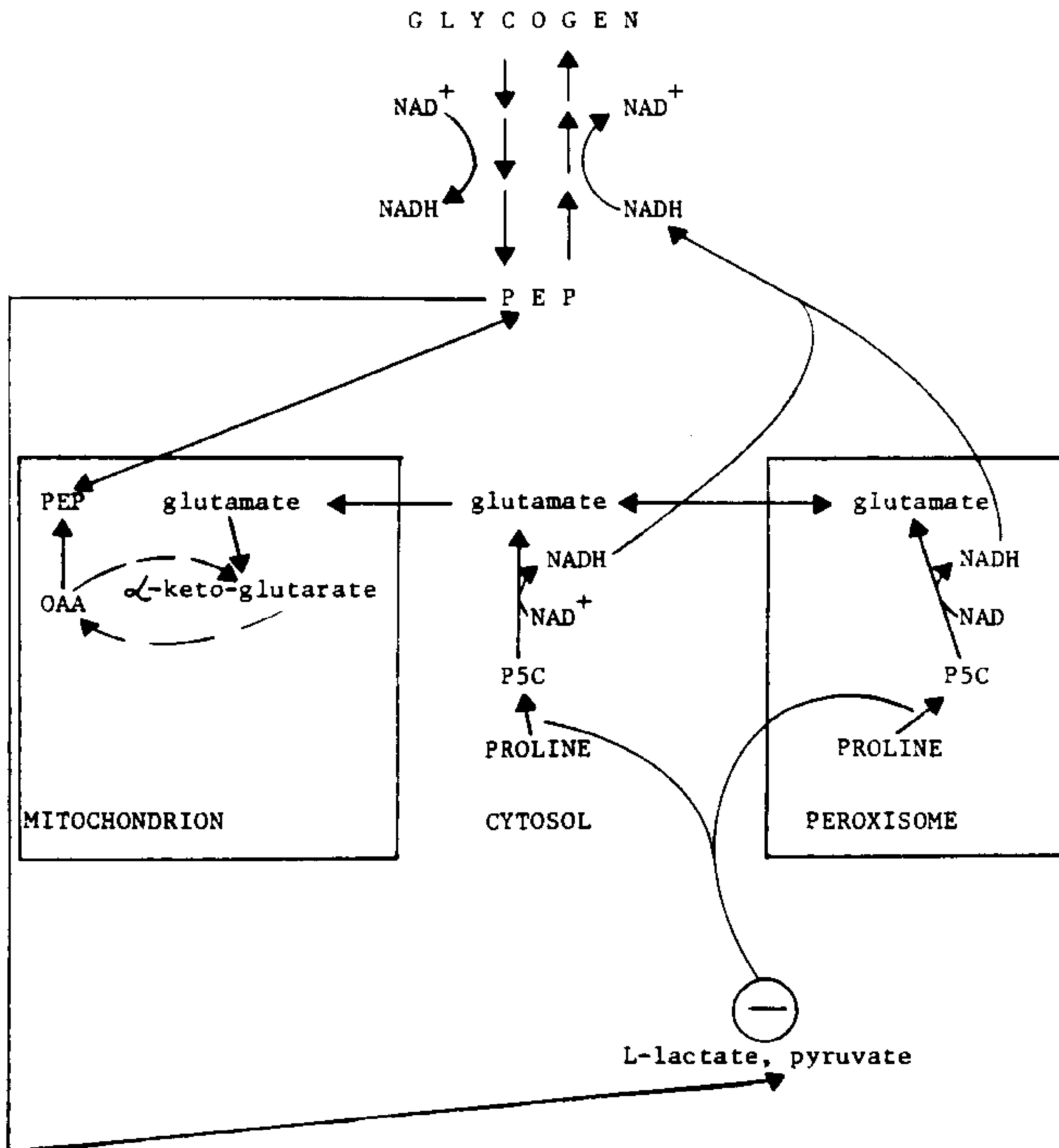
The second reaction is catalyzed by P5C dehydrogenase. In this reaction P5C is oxidized to glutamate (52,53). This reaction is NAD dependent--NAD is reduced 5 times faster than NADP (53). The equilibrium of this reaction lies greatly in favor of glutamate formation.

Both enzymes appear to be mitochondrial in rat liver (53). However, in many studies the procedure used (56) to obtain mitochondrial preparations does not differentiate between mitochondria and peroxisomes. The fact that peroxisomes contain a full set of enzymes for the β-oxidation of fatty acids (32), a pathway considered till recently to be mitochondrial, suggests that the apparent mitochondrial localization of the proline catabolizing system should be viewed with caution. In addition, Brunner et al. (57) showed that in rat liver approximately 50% of P5C-dehydrogenase is not particulate. Neither of the two enzymes, however, was assayed in Tetrahymena.

In our study it became apparent that, even though a large amount of extra ammonia and glycogen were produced in the presence of proline, neither the glycogen nor the ammonia could originate from proline alone. We suggest that the stimulation of the glyconeogenesis by proline can be explained by the NADH production during the conversion of proline into glutamate. Diagram 3 summarizes this hypothesis.

Diagram 3

Proposed mechanism of the stimulatory effect of proline on glyconeogenesis



A high level of cytosolic NADH is needed in glyconeogenesis (58). Williamson et al. (28) showed that oxidation of oleate in perfused rat liver stimulated gluconeogenesis from alanine. This was explained by an increased cytosolic NADH/NAD ratio due to oleate oxidation. The stimulatory action of proline can be explained in similar terms. NADH formed in P5C-dehydrogenase reaction should contribute to the efficiency of glyconeogenic process in the organism by speeding up the glyceraldehyde-3-phosphate dehydrogenase reaction.

Amino acids, to a large extent, appear to be the end products of proline metabolism in the ciliate. This is similar to rat liver where a dramatic accumulation of glutamate occurs during proline oxidation (59). The accumulation of the amino acid metabolites of proline in our incubation medium suggests strongly that the potent stimulation of glyconeogenesis with proline observed by us must be associated with the first two steps of proline metabolism, rather than with the utilization of its carbon chain. In order to substantiate this hypothesis it would be useful to establish the subcellular localization of the proline catabolizing system in the protozoan cells. However, in spite of numerous attempts to assay proline dehydrogenase in the subcellular fractions, no conclusive results were obtained, probably due to the instability of this enzyme.

In theory either cytosolic or peroxisomal P5C-dehydrogenase would provide cytosolic NADH. Peroxisomes, in contrast to the mitochondria, can secrete NADH (4) and thus may be a possible location for P5C dehydrogenase.

The available evidence indicates that the gluconeogenic stimulatory effect of proline in rat liver can be in part accounted for by the mechanism suggested (Diagram 3). It is of interest here to point to the inhibition of proline dehydrogenase by lactate and pyruvate. If the enzyme is associated with glyconeogenesis, such inhibition can be interpreted as a shutoff mechanism, when cells undergo excessive glycolysis.

To further support our hypothesis, it is important to note that Δ'-pyroline-5-carboxylate has been implicated already as a regulator of the cellular redox state. Phang et al. (60) showed that P5C is a potent stimulator of the hexose monophosphate-pentose pathway in cultured human fibroblasts. This stimulatory effect was also measured in human erythrocytes (61). The enzyme catalyzing the conversion of P5C to proline, proline reductase, is soluble and requires NADPH for its action (52). This enzyme is present also in Tetrahymena (18). The researchers suggested (60, 61) that the stimulatory effect of P5C is due to the generation in P5C reductase reaction of $NADP^+$, required by the two dehydrogenases of the hexose monophosphate-pentose pathway.

The stimulatory action of P5C described occurs in the reaction resulting in proline synthesis. However, proline synthesis and degradation seem to be well regulated.

In a recent paper on proline metabolism in mutant yeasts, Brandriss et al. (62) conclude that when P5C is derived from ornithine (via ornithine transaminase reaction) it is converted to proline by P5C reductase. If P5C is derived from proline, however, it

enters the P5C-dehydrogenase reaction. Thus in the yeast cell, the fate of P5C is determined by its chemical origin. Similar regulatory mechanism could be present in the Tetrahymena cells. Such a mechanism can involve different compartmentation of the enzymes involved in proline synthesis and degradation, or an aggregation of the enzyme proteins to prevent the release of intermediates into cytoplasm.

In addition, proline inhibits P5C formation from glutamate in Chinese hamster ovary cells (63). Thus in presence of proline, proline synthesis seems to be stopped by a feedback inhibition phenomenon. Thus the synthetic pathway, resulting in increased levels of $NADP^+$, or the degradative pathway, resulting in increase of cytosolic NADH, would occur according to the metabolic need of the cells.

The amino acid end products of proline metabolism are glutamate, aspartate, asparagine and glutamine. Contrary to some reports (51, 59) glutamate is not the major amino acid produced. The excretion of glutamine and asparagine could be the way this ammonotelic protozoan gets rid of extra ammonia. Glutamate, aspartate and asparagine were detected both inside the cell and in the medium, although the extracellular concentration was greater. Glutamine, however, was detected only in the incubation medium. This suggests a cell membrane-bound glutamine synthetase, converting glutamate to glutamine and transporting glutamine to the outside of the cell.

According to our hypothesis proline stimulates glyconeogenesis by elevating the cytoplasmic NADH level and thus stimulating the glyceraldehyde phosphate dehydrogenase step in gluconeogenesis. Since ammonia produced in response to added proline cannot be accounted for

by proline alone, it appears that the increase of NADH level in cytosol causes increased endogenous amino acid metabolism. Tetrahymena are known to have a large amount of free intracellular amino acids (19, 49) that could be used for glyconeogenesis. Increased protein degradation, however, cannot be excluded.

Under the conditions studied, Tetrahymena pyriformis, strain GL, seems to be unable to accomplish a net conversion of acetate to glycogen. Among lipid substrates tested, only Tween 40 had a significant stimulatory effect on glyconeogenesis. Neither Tween 80 nor lecithin affected glyconeogenesis of the ciliate.

Among amino acids tested besides proline, the best stimulants of glyconeogenesis were threonine, leucine and asparagine. By the use of dipeptides as substrates, phenylalanine and valine must be added to this list. Among these amino acids, threonine, leucine and phenylalanine are ketogenic. L-leucine is the most clearcut member of the group since it generates only Acetyl-CoA and its precursors. The glyoxylate bypass enzymes, then, should allow the net conversion of Acetyl-CoA into glycogen. Our data indicate that the glyconeogenic effect of leucine can be explained to a large extent by the conversion of carbon atoms of leucine into glycogen. Thus this amino acid, ketogenic in mammals, appears to be glycogenic in Tetrahymena due to the presence of a glyoxylate cycle. It should be pointed out, however, that a significant part of the available leucine is simply oxidized by the cells.

When cells of Tetrahymena are supplied with a mixture of proline and leucine, the glycogen increment obtained was determined to be

exactly additive. It thus appears that leucine and proline enter the protozoan cells by separate transport systems. Once inside the cells, leucine seems to augment the endogenous source of acetyl CoA for glyoxylate cycle. At the same time proline stimulates reversed glycolysis. It is also possible that leucine also supplies reducing power for reversed glycolysis. Thus both amino acids provide a complete and independent package for glyconeogenesis to occur.

The use of dipeptides as substrates for glyconeogenesis enabled us to learn several facts about the Tetrahymena cells. In the first place, dipeptides can help to overcome a permeability barrier for an amino acid. Thus, phenylalanine and valine are very poorly utilized by these cells. However, when bound to proline they can be utilized quite well for glyconeogenesis. This implies that Tetrahymena might possess a separate transport system or even systems for dipeptides. Such preference for dipeptides has been demonstrated in other systems (48). This transport system enables the uptake of the dipeptides into the cell. Secondly, proline dipeptides with N-terminal proline appear to be better substrates than C-terminal proline dipeptides. This could reflect either a specificity of transport or of an endogenous protease, responsible for the hydrolysis of the dipeptide bond. Post-proline cleaving endopeptidase was reported in human uterus and flavobacterium (64). Our data suggests that a similar enzyme might be present in Tetrahymena.

Tetrahymena are known to excrete proteases into the medium (4). In addition, a protease associated with the outer cell surface has

also been reported (65). The comparison of the effectiveness of amino acid mixtures vs. the corresponding dipeptide indicates, however, that the dipeptides are indeed hydrolysed inside the protozoan cells.

It is of interest to comment on the data obtained with methionine and its dipeptides. The inhibitory effect on glyconeogenesis exerted by methionine remains unexplained. However, our data indicate that this inhibitory effect results from the presence of methionine within the cell. Free proline blocked the entrance of free methionine but prolyl-methionine showed a marked inhibition of glyconeogenesis, in terms of the increase expected of its proline content. The inhibitory effect of methionine could be due to toxicity of methionine itself or any of its metabolites. In addition, methylation of a specific protein, RNA or lipid could be crucial to gluconeogenesis.

Because our results on transport of amino acids and peptides are based chiefly on indirect measurement (conversion to glycogen), they have only a limited value. The positive results are of course valid; the negative results may reflect a lack of metabolic utilization. In the specific case of Tetrahymena, however, the case of prolyl dipeptides constitutes a valuable practical advance in the study of its amino acid nutrition and metabolism.

In summary, in this work, protein glyconeogenesis was studied in _Tetrahymena pyriformis_. Because of the presence of glyoxylate bypass enzymes, amino acids that are ketogenic in mammals appear to be glucogenic in Tetrahymena. A glucogenic amino acid L-proline seems to stimulate glyconeogenesis primarily by supplying cytosolic NADH to

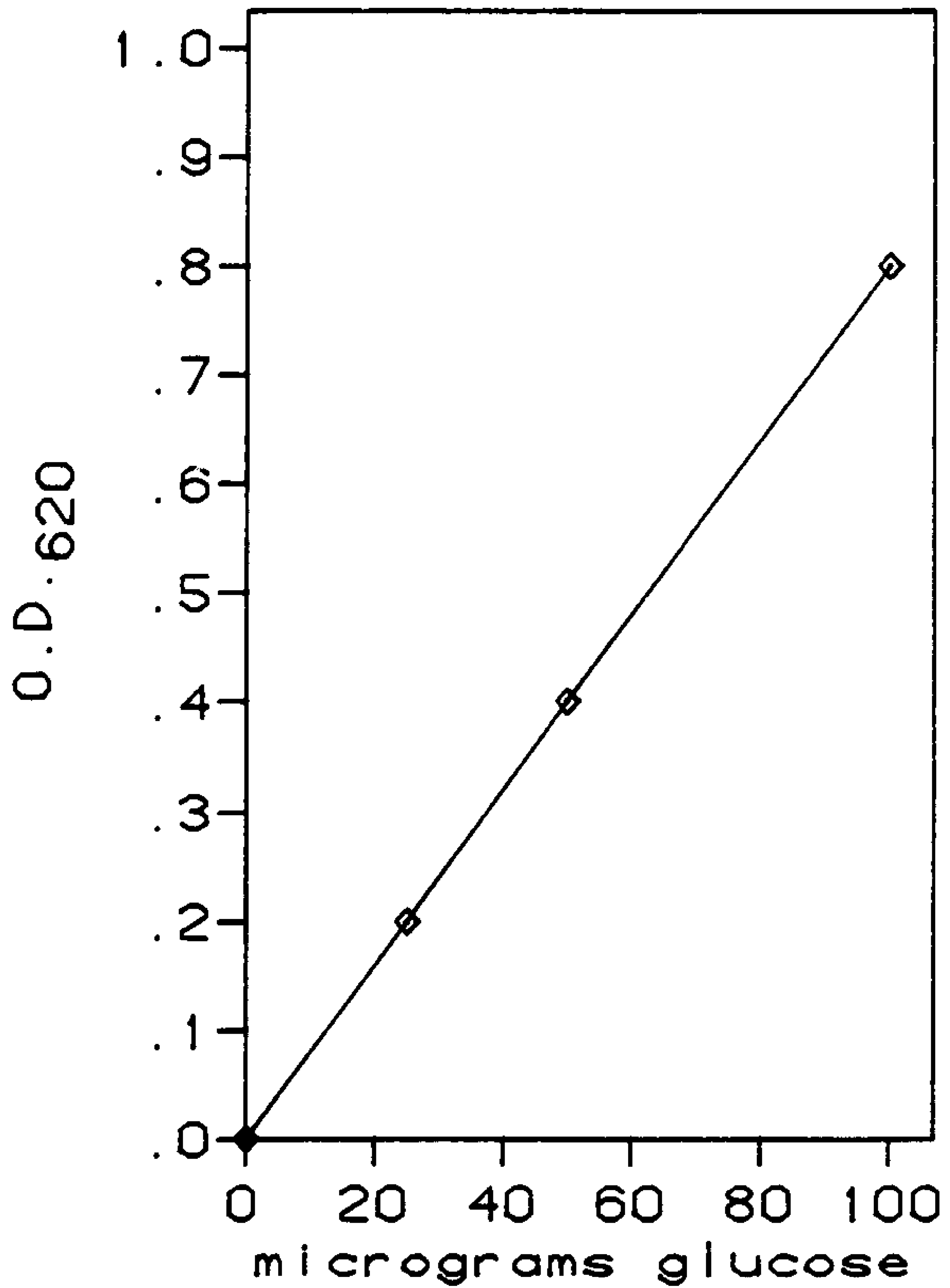
the process, and only to a small extent by direct contribution of its carbon atoms for glycogen synthesis.

In the Introduction (part B), we outlined an experimental framework to be followed in this study. As has been presented, to a large extent we have succeeded in completing the plan.

APPENDIX

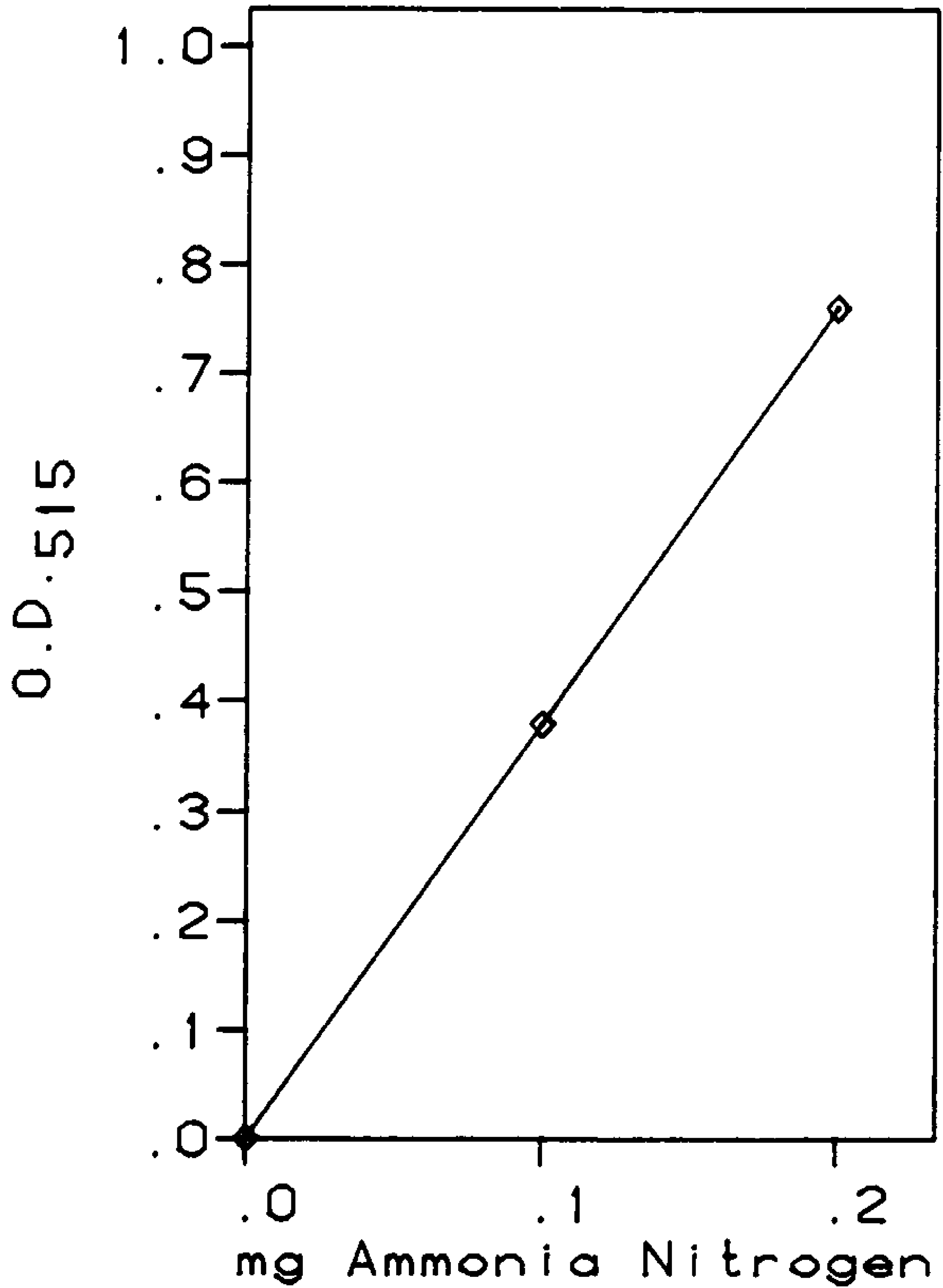
Appendix Figure 1

Standard curve for glucose determination by the Anthrone method



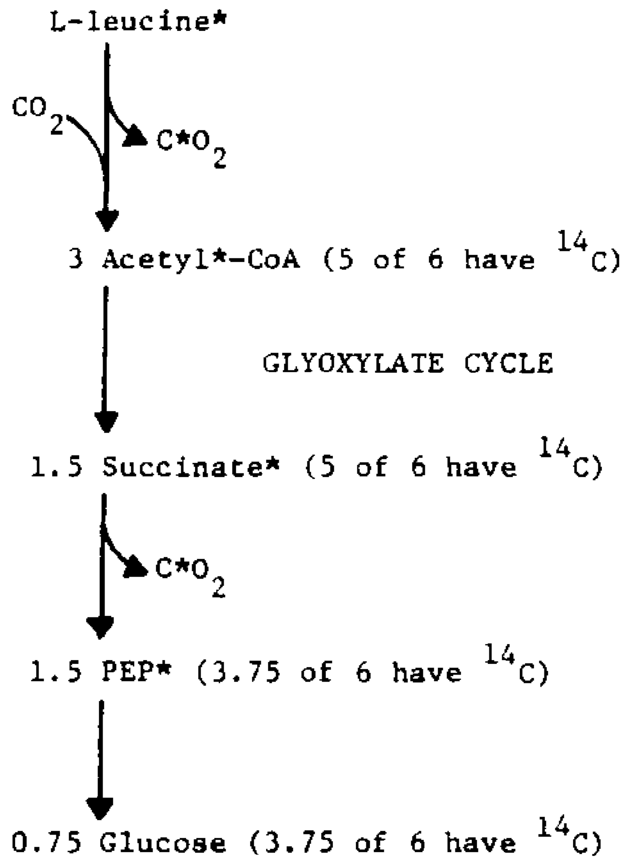
Appendix Figure 2

Standard curve for NH_3-N by Nesslerization



Appendix Diagram 1

The metabolic fate of L-leucine in presence of glyoxylate cycle



REFERENCES

1. Manners, D.J. and Ryley, J. F., Biochem. J., 52, 480-482 (1952).

2. Wagner, C., Ph.D. Thesis, University of Michigan, Ann Arbor, Michigan (1956).

3. Hogg, J. F., Ann. N.Y. Acad. Sci., 168, 281-291 (1969).

4. Hill, D. L., The Biochemistry and Physiology of Tetrahymena, Academic Press (1972).

5. Roth, J. S. and Eichel, H. J., Biol. Bull., 108, 308-311 (1955).

6. Scherbaum, O. H. and Levy, M., Path. Biol., 9, 514-517 (1961).

7. Hogg, J. F. and Kornberg, H. L., Biochem. J., 86, 462-468 (1963).

8. De Duve, C. and Baudhuin, P., Physiol. Rev., 46, 232-256 (1966).

9. Szabo, A. S. and Avers, C. J., Ann. N.Y. Acad. Sci., 168, 302-312 (1969).

10. Cooper, T. G. and Beevers, H., J. Biol. Chem., 244, 3514-3520 (1969).

11. Tolbert, N. E. and Yamazaki, R. K., Ann. N.Y. Acad. Sci., 168, 325-341 (1969).

12. Goodman, D. R. P., Davis, W. L. and Jones, R. G., PNAS, 77, 1521-1525 (1980).

13. Jones, C. T., BBRC, 95, 849-856 (1980).

14. Krebs, H. A., Speake, R. N. and Hems, R., Biochem. J., 94, 712-720 (1965).

15. Shrago, E. and Elson, C., Biochemistry and Physiology of Protozoa, Second Edition, Vol. 3, 87-312, Academic Press (1980).

16. Scherbaum, O. H. and Levy, M., J. Gen. Microbiol., 38, 221-230 (1965).

17. Reynolds, H., J. Microbiol., 104, 719-725 (1970).

18. Hill, D. L. and Chambers, P., BBA, 148, 435-447 (1967).

19. Wu, C. and Hogg, J. F., J. Biol. Chem., 198, 753-764 (1952).

20. Felig, P., Ann. Rev. Biochem., 44, 933-954 (1975).

21. Ross, B. D., Hems, R. and Krebs, H. A., Biochem. J., 101, 284-296 (1966).

22. Roth, J. S., Eichel, H. J. and Ginter, E., Arch. Biochem. Biophys., 48, 112-119 (1954).

23. Mavrides, C. and D'Iorio, A., BBRC, 35, 467-475 (1969).

24. Whitlow, K.J., D'Iorio, A. and Mavrides, C., BBA, 264, 440-446 (1971).

25. Mavrides, C., Can. J. Biochem., 51, 323-331 (1973).

26. Elson, C., Shrago, E., Sondheimer, E. and Yatvin, M., BBA, 297, 125-134 (1973).

27. Warnock, L. G and Van Eys, J., J. Cell. Comp. Physiol., 60, 53-59 (1962).

28. Williamson, J. R., Browning, E. T. and Schulz, R., J. Biol. Chem., 244, 4607-4616 (1969).

29. Hryb, D. J. and Hogg, J. F., Fed. Proc., 35, 1501 (1976).

30. Lazarow, P. B. and de Duve, C., PNAS. U.S.A., 73, 2043-2046 (1976).

31. Hryb, D. J. and Hogg, J. F., BBRC, 87, 1200-1206 (1979).

32. Osumi, T. and Hashimoto, T., J. Biochem. (Tokyo), 85, 131-139 (1979).

33. Muller, M., Ann. Rev. Microbiol., 29, 467-480 (1975).

34. Conner, R. L., Cline, S. G., Koroly, M. J. and Hamilton, B., J. Protozool., 13, 377-379 (1966).

35. Ryley, J. F., Biochem. J., 52, 483-492 (1952).

36. Hogg, J. F., personal communication.

37. Hogg, J. F., personal communication.

38. Patterson, M. S. and Green, R. C., Anal. Chem., 37, 854-857 (1965).

39. Koch, P. and McMeekin, S., Pract. Physiol. Chem., Hawk, P. B., Oser, B. L. and Summerson, W. H. The Blackstone Comp. Inc. 13th Edition, 1329 (1947).

40. Hogg, J. F., personal communication.

41. Clark, J. M. and Switzer, R. L., Exper. Biochem., second edition, 91-96, W. H. Freeman and Company (1977).

42. Weichselbaum, T. E., M. J. Pathol., 10, 40-44 (1946).

43. Dayhoff, M. O., Atlas of Protein Sequence and Structure 1972, National Biomedical Research Foundation, Washington, D. C. (1972).

44. Hoffman, E. K. and Kramhoft, B., Exp. Cell Res., 56, 265-269.

45. Rasmussen, L., Nature, 250, 157-158 (1974).

46. Muller, M. and Rohlich, P., Acta Biol. Acad. Sci. Hung., 12, 34-39 (1962).

47. Hoffman, E. K. and Rasmussen, L., BBA, 226, 206-210 (1972).

48. Barack, Z. and Gilvarg, C., Biomembranes, 7, 167-218 (1975).

49. Wu, C., Hogg, J. F. and Elliott, A. M., Fed. Proc., 11, 207-211 (1952).

50. Kidder, G. W., Dewey, V. C. and Heinrich, M. R., Exp. Cell Res., 7, 256-264 (1954).

51. Dewey, V. L. and Kidder, G. W., J. Protozool., 19(1), 50-53 (1972).

52. Adams, E. and L. Frank, Ann. Rev. Biochem., 49, 1005-1061 (1980).

53. Strecker, H. J., Methods Enzymol., 17B, 251-265 (1971).

54. Kramar, R., Enzymologia, 33, 33-37 (1967).

55. Scarpulla, R. C. and Soffer, R. L., J. Biol. Chem., 253, 5997-6001 (1978).

56. Hogeboom, G. H., Methods Enzymol., 1, 16-19 (1962).

57. Brunner, G. and Neupert, W., FEBS Letters, 3, 283-286 (1969).

58. Krebs, H. A. and Hems, R., Biochem. J., 93, 623-627 (1964).

59. Hensgens, H. E. S. J., Meijer, A. J., Gimpel, J. A. and Tager, J. M., Biochem. J., 170, 699-707 (1978).

60. Phang, J. M., Downing, S. J., Yeh, G. C., Smith, R. J. and Williams, J. A., BBRC, 87, 363-370 (1949).

61. Yeh, G. C. and Phang, J. M., BBRC, 94, 450-457 (1980).

62. Brandriss, M. C. and Magasanik, B., J. Bacteriol., 143, 1403-1410.

63. Smith, R. J., Downing, S. J., Phang, J. M., Ledato, R. F. and Aoki, T. T., PNAS, 77, 5221-5225 (1980).

64. Yoshimoto, T., Walter, R. and Tsuru, D., J. Biol. Chem., 255, 4786-4792 (1980).

65. Zdanowski, M. K. and Rasmussen, L., J. Cell Physiol., 100, 407-412 (1979).