#### THE UNIVERSITY OF MANITOBA

## STUDIES ON <u>TETRAHYMENA</u> <u>PYRIFORMIS</u> <u>W</u>. EFFECTS OF CARBOHYDRATE ON GROWTH AND USE OF THE ORGANISM IN PROTEIN QUALITY EVALUATION.

by

NICHOLAS C. CUMBERBATCH

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# STUDIES ON <u>TETRAHYMENA PYRIFORMIS</u> <u>W</u>. EFFECTS OF CARBOHYDRATE ON GROWTH AND USE OF THE ORGANISM IN PROTEIN QUALITY EVALUATION.

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## NICHOLAS C. CUMBERBATCH

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

## MASTER OF SCIENCE

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## DEDICATED

TO MY PARENTS VINCENT AND LOUISA CUMBERBATCH AND MY SISTERS AND BROTHER



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### ABSTRACT

The effects of carbohydrates on the growth response of <u>Tetrahymena pyriformis</u> W were investigated. The organism also was applied to protein quality evaluation, amino acid assays, and amino acid supplementation studies. Test media were inoculated with cells grown in continuous culture with the aim of standardizing the physiological state, age and size of the inoculum. Mean cell sizes, cell counts and total population volumes (= mean cell volume x cell count) at 12 hr intervals up to 120 hr were measured using the Coulter Counter. Growth curves (log<sub>10</sub> total population volume vs time) were plotted and growth rates were determined.

The study of the effects of carbohydrates on the growth response of the organism was divided into two parts. Part (a) dealt with the effect of varying the concentration of glucose and starch (0 - 20.0 mg/ml.) on the mean cell volume, maximum cell counts, maximum total population volume and growth rate of <u>Tetrahymena pyriformis</u> W grown in casein, whole egg protein and casein hydrolysate media at the isonitrogenous level of 0.3 mgN/ml. Statistical analyses of the results revealed that there was no significant difference (P = 0.05) between the growth-stimulatory effects of glucose and starch. Significant effects were produced by varying the carbohydrate concentration and the protein source and also by the protein/type of carbohydrate and protein/concentration of car-

bohydrate interactions. It was noted too that unlike starch, the high concentration of glucose, 20.0 mg/ml. increased the lag phase of the growth cycle. In Part (b) the effects of varying four different carbohydrate sources, glucose, starch, dextrinand a glucose/starch (1:1) mixture, at the same concentration (10.0 mg/ml.), were studied when the form of the nitrogen source was either the intact whole egg protein or an amino acid mixture of similar pattern as the intact protein. The results indicated that varying the carbohydrate source significantly affected the growth response of the organism. Furthermore, the simultaneous presence of glucose and starch stimulated optimal growth responses in both the intact protein and the free amino acid media. Several hypotheses, such as osmotic effect, mechanical stimulation of particles and differences in the rates of uptake and availability of glucose and amino acids, were discussed for explaining these effects.

The growth response of the organism was used to evaluate the nutritive values of different protein sources relative to that of casein. The quality of these proteins was expressed as the Relative Nutritive Value, RNV (casein = 100). Two sets of proteins were evaluated, cereals and non-cereal vegetable and animal protein isolates and concentrates. The RNVs of the cereals (wheat and triticale) were determined using maximum cell counts obtained with a haemocytometer. The RNVs of the protein isolates and concentrates (faba bean, zein, soya bean, casein, whole egg, herring and cod) were determined using the maximum total population volumes obtained with the Coulter Counter. The RNVs of the cereals correlated highly (r = 0.96, P = 0.05) with the published Protein The RNVs of Index values from rat feeding experiments. both fish protein concentrates, herring and cod frames, and those of the faba bean proteins did not agree with literature rat values. Reasons for these discrepancies were discussed. On the other hand, the RNVs of the other proteins correlated reasonably well with the published and expected results. It was suggested from the study that maximum total population volume, instead of cell count at merely one incubation period, would more accurately estimate protein quality.

The amino acids lysine, threonine and methionine plus cystine contents of the cereals and isoleucine, lysine, threonine and tryptophan contents of casein, whole egg and faba bean were determined. Generally, results compared favourably with chemically-determined values. Study of the effects of supplementation of faba bean with DL-methionine, of cod and herring with L-tryptophan and of the cereals with L-lysine hydrochloride and DL-methionine produced variable results.

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# INTRODUCTION

#### INTRODUCTION

Ever since the eighteenth century, when it was recognized that proteins of diverse origin differ considerably in nutritive value, workers have been actively engaged in the field of protein quality research. Methodology by which nutritive values are determined, has evolved considerably over the years: from the simple animal feeding trials which estimate protein value in terms of change in body weight to relatively more complex expressions such as those involving changes in plasma amino acids. Protein quality evaluation methods have been developed for two main reasons: 1) to provide a procedure for ranking proteins according to their nutritional value, 2) to predict the efficiency of utilization of proteins as sources of nitrogen and amino acids for meeting the amino acid requirements of man and animals.

The quality of a protein is dependent on its amino acid composition, its digestibility and the amounts of its constituent amino acids that are utilized. In addition, the quality or nutritive value of a protein is dependent on the species, strain or method used for its evaluation and different results may be obtained for the same protein by different laboratories using the same method.

Established biological methods, using laboratory

animals such as mice, rats and chicks are time-consuming, laborious and expensive. Although refinements are needed to improve their reproducibility, the major need is for a rapid and inexpensive procedure. Microbiological methods have shown promise, but have proved much less simple than was anticipated when they were introduced. One such method involves the use of the ciliated protozoan, Tetrahymena pyriformis W.

The usefulness of the <u>Tetrahymena pyriformis</u> method, and indeed of all biological methods of protein quality evaluation, depends on successfully ensuring that, under experimental conditions, protein is the only factor limiting the response chosen to be measured. Therefore, knowledge of the organism's response to components of the growth medium, other than protein itself, is important to the assay procedure. Since protein can be used by the organism for energy production as well as for the production of new cell material, the presence of another energy source is required in the medium to spare protein from being "wastefully" utilized.

Generally carbohydrate has been used as the energy source in the assay medium, being added either directly or indirectly as part of the intact protein source. Most intact proteins contain varying proportions of different types of carbohydrates and also the type of exogenous carbohydrate source added to the medium has varied. If the growth response of Tetrahymena pyriformis W to protein

is affected by the type and amount of carbohydrate present in the growth medium, then any protein quality evaluation must take this into account. It is important also to determine whether the effect of the type of carbohydrate varies with the form in which the nitrogen is supplied in the medium (intact protein vs free amino acids), since amino acid determination usually involves comparisons of growth responses of the organism to intact proteins and crystalline amino acid mixtures.

The objectives of this study then are: -

- To determine the effect of different types and concentrations of carbohydrate on the growth responses of <u>Tetrahymena pyriformis</u> W in media containing different types of nitrogen sources.
- To demonstrate the usefulness of the organism for protein quality evaluation including amino acid availability determinations.

LITERATURE REVIEW

#### LITERATURE REVIEW

Since accepted methods for protein evaluation using laboratory animals are costly, too time-consuming and laborious for routine use in screening large numbers of samples, increasing attention has been given to microbiological methods. Strains of bacteria including <u>Steptococcus faecalis, Streptococcus zymogenes</u> and <u>Leuconostoc mesenteroides</u> and the protozoan, <u>Tetrahymena</u>, have been exploited.

S. faecalis (Halevy and Grossowicz, 1953) and L. mesenteroides (Horn et al, 1954) have been used as assay organisms with the limitation that test proteins had to be subjected to enzyme or acid hydrolysis. Although Halevy and Grossowicz (1953) using pancreatic protein hydrolysates, obtained results generally comparable with rat growth values; two anomalies led one to question the validity of the method. Egg albumen was found to have essential amino acid deficiencies not encountered in higher animals and the response of the microorganism to gelatine, though lacking in tryptophan, was appreciable. Horn et al (1954) used L. mesenteroides P. 60 to determine the effect of heat treatment on the nutritive value of enzyme and acid hydrolysates of cottonseed protein. Their results agreed reasonably well with animal assays but the limited application of the method affected its acceptance as a general procedure.

Rogers et al (1958) using a variety of proteins of both plant and animal origin evaluated these bacteriological methods by comparing results with protein efficiency ratios (PER) determined using rats. As well as noting discrepancies between bacteriological estimates and rat growth data, these workers observed that lysine and arginine were the most limiting amino acids for S. faecalis and L. mesenteroides for all the protein hydrolysates studied. Ford (1960), in an attempt to eliminate possible effects of prolonged enzyme or acid pretreatment on protein quality, introduced the use of S. zymogenes, a highly proteolytic bacterium. Preliminary results correlated well with animal growth values. However, in practice this method is limited since S. zymogenes requires glutamate but lysine, threonine and phenylalanine are non-essential amino acids. Therefore the bacteriological methods so far investigated had proven to be unsatisfactory for routine use in protein quality evaluation.

The proteolytic capacity of <u>Tetrahymena</u> was first reported by Lawrie (1957) and later made use of in protein quality assays by Rockland and Dunn (1946, 1949). The proteolytic system has been studied (Viswanatha and Liener, 1956; Dickie and Liener, 1962; Smith, 1961) and suggestions have been made for increasing the proteolytic activity by sulphydryl activation (Viswanatha and Liener, 1956; Maciejewicz, 1972). Also Shorrock and Ford (1973)

have shown that pretreatment of test proteins with papain yielded results which correlated more closely to those obtained with animal assays. Nevertheless, the majority of papers describing the use of <u>Tetrahymena</u> in protein quality evaluations have reported acceptable values without proteinase activation or enzyme treatment.

Tetrahymena pyriformis has been studied by several workers with the objectives of elucidating its nutritional requirements and assessing its merit as an assay tool in protein quality determinations. Much of the information relevant to the application of the organism to nutritional studies stems from the comprehensive investigation of Kidder and Dewey (1951). These workers established that, in addition to its proteolytic powers and its ability to be cultured in chemically defined media, <u>Tetrahymena pyriformis</u> strain W has the additional advantage of requiring the same essential amino acids as higher animals including man.

A major problem inherent in protein quality evaluation with <u>Tetrahymena</u> has been the lack of an unequivocal method for estimating growth, or more specifically the amount of cellular protein synthesized in media containing intact proteins in solution or suspension. In <u>Tetrahymena pyriformis</u> assays, protein quality has been expressed as the growth response of the organism to the test protein relative to a standard such as casein or whole egg protein. Different methods

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have been proposed both for assessing the growth response and for expressing the Relative Nutritive Value (RNV). Rockland and Dunn (1949) used acid production by Strain H as an index of growth. A colorimetric method based on the determination of the red triphenylformasan formed by the enzymic conversion of colorless 2, 3, 5 triphenyltetrazolium chloride was proposed by Anderson and Williams (1951) and later modified by Pilcher and Williams (1954). However, the colorimetric method has been rejected because of inaccuracies demonstrated by Fernell and Rosen (1956) and Jambor (1955). Similarily, turbidimetric methods have proven useless because of changes in optical density associated with unutilized or undigested food material and variation in the glycogen content of cells (Levy and Wasmuth, 1970). On the assumption that the composition and average dry weight of the organism is independent of the protein source, Rosen and Fernell (1956) reasoned that the amount of cellular protein synthesized will be proportional to the number of organisms in the culture. Thus microscopic cell counts using a haemocytometer have been used by several workers (Fernell and Rosen, 1956; Teunisson, 1961; Stott et al, 1966; Helms and Rolle, 1968). Futile attempts to separate Tetrahymena cells from the medium, by differential centrifugation or by electromagnetic techniques (Fernell and Rosen, 1956), had ruled out the use of conventional methods such as dry weight, total

nitrogen or protein nitrogen synthesis. Recently, however, Teunisson (1971) has proposed an efficient elutriation methos for separating cells from residual food matter, and the use of the Coulter Counter for the faster electronic counting of cells. The Coulter Counter (Coulter, 1956) automatically counts and sizes thousands of microscopic particles with great precision and consistent reproducibility, essentially one-by-one, in a few seconds. Its merits, relative to the haemocytometer, have been evaluated by several workers such as Brecher et al (1956), Mattern et al (1957) and Richard et al (1959). In a study on the growth and feeding kinetics of Tetrahymena pyriformis, Curds and Cockburn (1971) established a linear relationship between cell volume and dry mass (r-0.95; Mass = 0.072 Volume). Based on this relationship, these workers used the product of the mean cell volume and the cell numbers/ml, as an estimate of ciliate biomass.

Throughout the past few years different approaches have been suggested for expressing the Relative Nutritive Value (RNV) of a protein source using casein or whole egg as the standard. Fernell and Rosen (1956) adopted a cell-count/NH<sub>3</sub> - nitrogen ratio as an index of protein utilization. Subsequently, Rosen (1960) observed that this ratio was not solely a function of protein utilization, proposed the use of the cell-count only. Teunisson (1961) estimated nutritional quality as an index: the ratio of

the regression coefficient of growth on the nitrogen content of the test protein to that on the nitrogen content of casein. On the premise that the maximum cell count for each protein is greatest during the logarithmic growth phase, Helms and Rolle (1968) suggested a log formula for calculating RNV: RNV=Log<sub>10</sub> (cell count for) -  $\frac{\text{Log}_{10}}{(\text{test protein})} - \frac{\text{Log}_{10}}{(\text{inoculum})} \times 100$  $\frac{\text{Log}_{10}}{(\text{cell count for})} \times 100$ 

Helms and Rolle (1968), Rolle and Eggum (1971) and Rolle (1973) applied this formula, which is the equivalent of the comparison of growth rates with the test proteins measured after four days of incubation. However, the organisms had completed the logarithmic growth phase by 36 hrs. (Helms and Rolle, 1968) and would have been well within the stationary phase by the fourth day. On the other hand this formula has the advantage of taking into consideration the usually-variable inoculum cellcount.

An important requirement for protein quality testing is the presence of an adequate energy source to spare protein. <u>Tetrahymena pyriformis</u> has been shown to obtain energy from amino acids and protein when other energy sources are not present in the medium (Hutner, 1963; Cox <u>et al</u> 1968). Thus in the assays with <u>Tetrahymena</u> the proteins have been fat-extracted and carbohydrate has been used as the sole source of energy. Different

carbohydrates have been investigated as energy sources. Holtz et al (1959) using Tetrahymena pyriformis type II var. I and chemically defined media found that acetate, glycerol, glycerolphosphate, lactate and pyrurate stimulated growth of the organism but not to the same extent as glucose. Cellobiose, dextrin, starch and glycogen were found to be as effective as glucose. Kidder and Dewey (1945), using various strains of Tetrahymena found that strain W fermented glucose, fructose, mannose, maltose, dextrin, glycogen and starch at the 0.5% level in a 1% proteose peptone In addition they noted that this strain could medium. ferment cellobiose in a 2% proteose peptone medium but not in a 1% medium. Strain W, unlike strains E and H, failed to ferment galactose. None of the strains of Tetrahymena was found to utilize other disaccharides, other polysaccarides, pentoses or polyhydric acids. Lack of ability to ferment a particular carbohydrate does not mean, however, that the compound is not a substrate for cellular enzymes. Cell-free extracts of Tetrahymena pyriformis not only catalyze the hydrolysis of maltose and starch but also of cellobiose and sucrose (Archibald and Manners, 1959). The lack of the cell's ability to ferment the latter two compounds was thought to be due to a permeability problem.

Each of the enzymes involved in the interconversion of glycogen and pyruvate (figure 1) are present in

Figure 1: Pathways of carbohydrate metabolism



<u>Tetrahymena pyriformis</u> (Warnock and Van Eys, 1962). Materials other than glycogen are degraded by the glycolytic pathway. Dextrin is cleaved, perhaps both hydrolytically and phosphorylytically, to monosaccharide units (Reynolds, 1969); and the monosaccharides, glucose, mannose, and fructose are phosphorylated prior to their entry into the reaction sequence (Seaman 1950). All of the enzymes of the Krebs (tricarboxylic acid) cycle are present in Tetrahymena pyriformis (figure 1).

Studies on the carbohydrate metabolism of <u>Tetrahymena</u> (Van Neil <u>et al</u>, 1942; Warnock and Van Eys, 1962) showed that glucose was oxidized to CO<sub>2</sub> in the presence of oxygen; but under anaerobic conditions succinate, lactate and acetate were produced according to the following reactions:

1. Aerobic: Glucose +  $60_2 \longrightarrow 6C0_2 + 6H_2^0$ 2. Anaerobic: 2 Glucose +  $C0_2 \longrightarrow 2$  succinate + lactate 3. Anaerobic without  $C0_2$ : Glucose  $\longrightarrow 2$  lactate

<u>Tetrahymena</u> differs from mammals in its carbohydrate metabolism in that it possesses a glyoxylate cycle (figure 1) to permit the net conversion of lipid to carbohydrate. The findings by Ryley (1952) that the respiratory quotient of <u>Tetrahymena pyriformis</u> is low, implied that lipid, rather than carbohydrate, is oxidized to supply cellular energy. Lipid thus appears to be more important than carbohydrate for energy production of normally - growing cells. Glycogen accumulates at the expense of intra-

cellular lipids (Warnock and Van Eys, 1962) even in a non-nutritive medium. Obviously there can be no net synthesis of glycogen from lipid which is metabolized by the Krebs cycle, since two molecules of CO<sub>2</sub> are produced for each molecule of acetate utilized. This is the situation in mammalian cells.

A few attempts have been made to study the comparative effects of different types of carbohydrates on the growth of Tetrahymena pyriformis W with variable results. Reynolds and Wragg (1962) studied the effect of different levels of dextrin and glucose on the growth of the organism as measured by optical density (OD) and cell protein determination in media containing an amino acid mixture. The results showed that both the rate of growth (2-7 day period) and the maximum growth attained were greater in the medium with dextrin than with glucose at concentrations 0.5% (w/v). In addition they observed that in both chemically defined and crude media the average volume of individual cells was 1.5 to 2 times greater in the presence of the polysaccharide compared to the monosaccharide. The apparent efficiency of conversion of media nitrogen to protein nitrogen, as indicated by the  $NH_3$ - N/protein - N ratio, was much greater in media with dextrin than with glucose. In agreement with previous reports (Kidder and Dewey, 1949; Rosen and Fernell, 1956), they found that high glucose concentrations 3% (w/v) inhibited growth of Tetrahymena pyriformis in media

containing 0.3 mgN/ml but high concentrations of dextrin did not produce the same effect. Thus based on these results, Reynolds and Wragg (1962) concluded that protein was more efficiently utilized by <u>Tetrahymena</u> <u>pyriformis</u> W in the presence of a polysaccharide than when a monosaccharide was used.

Seemingly contradictory results were obtained by Stott et al (1963) who compared starch and glucose in intact protein media. Starch at 2% (W/V) and glucose at 0.75 - 1.5% were compared over the range of 0.1 -0.5 mgN/ml. Significantly higher microscopic cellcounts were produced in glucose compared to starch media. Stott et al (1963) therefore recommended use of glucose instead of starch, as a more suitable energy source for routine assays. Estevez (1962) studied the effects of glucose, maltose and dextrin with two nitrogen sources, an amino acid mixture and casein, on the growth of Tetrahymena pyriformis as measured by the dye reduction The carbohydrates were tested at 0.5 - 2.5%method. (W/V) in media at nitrogen level of 0.276 mg/ml. In the casein medium the growth response was the same in varying concentrations of glucose, maltose and dextrin. In the amino acid mixture the response was similar with glucose and maltose as energy source but was lower with dextrin. Also, growth was repressed with high levels of glucose in the undenatured protein medium but not in the amino acid medium. The growth of the organism appeared to be

dependent, not only on the nitrogen source, but also on the type and amount of carbohydrate. The results of Estevez (1962) are at variance with those of Reynolds and Wragg (1962). The latter used an amino acid mixture and observed higher growth responses in terms of optical density (OD) with dextrin than glucose at similar nitrogen and carbohydrate levels after identical incubation periods. They obtained the following results:

Glucose: OD = 0.41, Dextrin: OD = 0.61 (Reynolds and Wragg, 1962); Glucose: OD = 0.56, Dextrin: OD = 0.37, (Estevez, 1962).

Reddy (1965) showed a higher growth response with glucose, compared to either dextrin or starch in different native and amino acid media, thus confirming the findings of Estevez (1962) and differing from those of Reynolds and Wragg (1962). Reddy (1965) observed that when with the amino acid medium glucose could not be interchanged with starch or dextrin and recommended that comparisons of amino acid mixtures and intact proteins could only be done directly if glucose is the carbohydrate source.

Generally, <u>Tetrahymena pyriformis</u> has been applied to the evaluation of large numbers and types of protein samples with <u>Tetrahymena</u> results agreeing fairly well with the animal assay indices (Rosen and Fernell, 1956; Teunisson, 1961; Reynolds, 1964; Baum and Haenel, 1965; Kamath and Ambegaokar, 1968; Helms and Rolle, 1968; Rolle and Eggum, 1971). A few reports would seem to indicate

the unsuitability of this assay method in certain cases, viz., low RNVs compared to animal values for blood-meal and grass-protein concentrates (Rosen and Fernell, 1956; Maciejewicz, 1972) and for fish protein concentrates (Celliers, 1961; Rosen et al, 1962; Boyne et al, 1967); overestimated protein qualities of samples with low contents of sulphur-containing amino acids (Rolle and Tetrahymena pyriformis assay has also Eggum, 1971). been applied to the control of oilseed processing (Teunisson, 1961); the formulation of mixed feeds (Rosen et al, 1962; Maciejewicz, 1972); measurement of available amino acids and amino acid supplementation effects (Stott and Smith, 1966; Boyne et al, 1967; Rosen et al, 1962) and the effect of processing temperatures on the total and available amino acids (Kondos and McClymont, 1972).

## MATERIALS AND METHODS
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# ORGANISM

The organism has been variously described as <u>Tetrahymena pyriformis</u>, <u>Tetrahymena geleii</u> and <u>Glaucoma</u> <u>pyriformis</u>. For this study <u>Tetrahymena pyriformis</u> strain W was obtained from the American Type Culture Collection (ATCC #10542). A fresh stock culture was obtained every four months.

#### Nitrogen Sources

The nitrogen sources (Table I) were intact protein concentrates or isolates, cereals, a protein hydrolysate and a free amino acid mixture; (the amino acid compositions are shown in Appendix Tables I and II):

- i) animal protein: casein, whole egg protein, herring protein concentrate and cod frames.
- ii) plant protein: faba bean protein concentrate,soy protein concentrate, natural vegetable protein(nvp, a faba bean protein isolate) and zein.

iii) Cereals: CIMMYT Wheat and triticale.

iv) protein hydrolysate: Casamino Acids (Difco), acid hydrolysate of casein.

v) free crystalline amino acid mixture (Appendix Table III, <sup>1</sup>Solution F (requirement pattern of Tetrahymena pyrifo<u>rmis</u> W). The essential amino

<sup>1.</sup> Private communication, Dr. E. B. Smith, Foods and Nutrition Department, University of Manitoba.

Table 1

Protein Samples: Nitrogen and Pro	otein conte	nts and Sourc	e
Protein	% Nitroge	n % Protein (Nx6.25)	Source .
Casein, Vitamin free	14.5	90.7	Nutritional Bio- chem. Inc. Cleveland Ohio
Zein	15.1	84.4	
Whole Egg Protein	11.4	71.3	General Biochem. Inc. Chagrin Falls, Ohio
Faba Bean Concentrate	10.9	67.8	Prairie Regional Lab Nat. Res. Coun. Saskatoon.
Faba bean isolate (Cerebos vegetable protein, nyp)	13.7	86.9	Lord Rank Research Centre, High Wycombe England
Soy Protein concentrate GL301	11.2	70.0	Griffith Labs, Ltd. Ontario
Herring protein concentrate	13.6	85.0	Fisheries and Marine Service, Halifax.
Cod frames	12.0	75.0	II.
Casamino Acids (acid hydro- lysate of casein)	9.8	61.3	Difco Labs. Detroit, Michigan.
CIMMYT Cereals: -	% Nitrogen	% Protein (Nx5.7)	
4859 7-Cerros Wheat	1.95	11.1	Centro Internacional De Mejoramiento De Maiz y Trigo,
4860 Inia- Wheat	1.98	11.3	(International Maize
4861 PM-132-triticale	2,56	14.6	and wheat Improvement Centre)
4862 PM-2-triticale	2.60	14.8	CIMMYT cereals were supplied by Dr. E.T. Mertz, Dept. of Piochemistry Purdue
4863 PM-15-triticale	2.74	15.6	University

\* Triticale, a new wheat-rye hybrid, has been suggested as a potentially important food for humans in that the lysine and protein contents have been found generally higher than that of wheat.

acid requirements which were derived individually according to the procedure of Stott and Smith (1966) and the non-essential amino acids were added to simulate the pattern in whole egg.

#### Carbohydrates

The carbohydrates used were D (+) glucose (British Drug House), dextrin (Matheson, Coleman and Bell) and soluable starch (Mallinckrodt). These reagent-grade carbohydrates were found to be practically nitrogen-free by the Kjeldahl test.

# Culturing of Tetrahymena pyriformis W

a) <u>Stock Culture</u>: Stock cultures of Tetrahymena <u>pyriformis</u> W were maintained at 25<sup>0</sup>C. in 50 ml medicine bottles containing 10 ml nutrient broth of the following composition as recommended by ATCC:

Proteose peptone	(Difco)	0.5 g/100 ml
Bacto tryptone	(Difco)	0.5 g/100 ml
к н <sub>2</sub> ро <sub>4</sub>		0.012 g/100 ml
K <sub>2</sub> HPO <sub>4</sub>		0.050 g/100 ml

The pH was adjusted to 7.1 with  $K_2HPO_4$  and the medium was autoclaved at  $121^{0}$ C. for 10 min. Cultures were transferred weekly by sterile Pasteur pipette. Axenity was tested for routinely by plating on trypticase soy agar (Baltimore Bielogical Laboratories) incubating for up to four days at  $37^{0}$ C. and examining for the presence of contaminating organisms.

b) Continuous Culture: The organism was maintained

in continuous culture to provide a ready supply of cells that were of reasonably uniform population and in a steady state of metabolism. The continuous culture of <u>Tetrahymena</u> <u>pyriformis</u> W was used in an attempt to further standardize the procedure.

The design of the low-cost continuous culture apparatus (figure 2) was based on the suggestions of Baker (1968). The basic requirements were specified by Herbert <u>et al</u> (1965): a calibrated growth vessel, a means of keeping the volume of culture constant, a mechanism for delivering a steady flow of medium, an efficient system of agitation and means of controlling the temperature and the gas flow rate. The growth vessel was made of glass and autoclavable, nontoxic silicone rubber tubing was used for making connections. A Weir-type overflow provided culture volume control. Large 10-litre glass bottles were used for the medium and effluent vessels.

The medium used was a growth-limiting dilution of the original maintenance medium instead of the more expensive chemically defined medium:

Proteose Peptone	3.0 g/litre
Bacto Tryptone	3.0 g/litre
к н <sub>2</sub> ро <sub>4</sub>	0.12 g/litre
K <sub>2</sub> HPO <sub>4</sub>	0.50 g/litre
Penicillin G (Na salt)	0.031 g/litre
Streptomycin sulphate	0.050 g/litre
The medium and the partly	disassembled chemostat

Figure 2: Continuous Culture Apparatus



were sterilized at 121<sup>0</sup>C. for 20 min. in an autoclave. After cooling, the apparatus was assembled asceptically and the medium was pumped into the growth vessel to about 2/3 of its operational volume and the pump then stopped.

A 2.5 ml. inoculum of a three day pure batch culture of <u>Tetrahymena pyriformis</u> W was introduced asceptically into the growth vessel with a sterile hypodermic syringe and 18 G 1½ needle. Then the stirrer was started together with the air-flow, and when growth had become established, the medium flow was started.

The flow rate (f) was determined by clipping off the growth vessel and using a stop-watch to measure the rate of ascent of the medium up the pipette flow-meter. Temperature was controlled indirectly by a water-bath surrounding the growth vessel. An air-flow rate of 400 ml. air/min. was monitored using an air-flow meter attached to the air line.

Frequent samples of the continuous culture were taken for cell counting and sizing, for contamination tests, pH measurements and microscopic observations. The technique developed for sampling consisted of unclipping the tube between the growth vessel and the sampling port and clipping the tube from the Weir overflow. The rising air-pressure forces some of the culture into the sampling port. The air tube was then unclipped and the sampling tube reclipped. A cell concentration of approximately 70,000 cells/ml. was maintained at a dilution rate (f/v) of 0.18 hr.-1. The chemostat was

operated for 4 - 6 weeks or until it became contaminated. Basal Medium

Stock solutions A, B, C, and D (100 x final concentration) were prepared as outlined in Appendix Table IV. Solution E, which was 5 x final concentration, was freshly prepared just before use from solutions B, C, and D using 1 ml. of each per 20 ml. of solution E and the appropriate weights of guanylic acid (sodium salt), adenosine  $-2^1 - (3^1)$  - phosphoric acid, cytidylic acid and uracil given in Appendix Table IV. This solution was adjusted to pH 8.2. Solutions A, B, C, and D were stored in the freezer until required for use. Then they were thawed and kept temporarily at refrigerator temperature. These stock solutions were prepared freshly every 4 - 6 weeks.

#### Preparation of Test Materials

The test materials were defatted by hexane-extraction for 18 hr. in a Soxhlet apparatus and then dried overnight in a vacuum oven. The dried, defatted material was then ground in a Wiley Mill to pass through a 60-mesh BS sieve. The fat-extraction and grinding were omitted for protein samples already defatted and powdered.

The nitrogen contents of the ground test materials were determined by the macro-Kjeldahl method (AACC 1969). Suspensions of test materials were prepared to give a nitrogen content of 1 mg N/ml. and the pH was adjusted to 8.2 with 1 N NaOH and refrigerated overnight. After warming to room temperature, the pH was readjusted to 8.2. The free amino acid mixture, Solution F, was prepared as outlined in Appendix Table III to provide 3 mgN/ml. 25 ml portions of Solution F, adjusted to pH 7.1, were stored in a freezer for up to four week periods.

## Assay Procedure

The general assay procedure was based on the method of Stott <u>et al</u> (1963). Assays were performed in duplicate in 50 ml. Erlenmeyer flasks. A final medium volume of 10 ml. was made up in the following manner:

1. Solution E, freshly prepared - 2 ml.

2. Nitrogen source - the volume of the nitrogen source provided a total nitrogen content of 3 mgN/10 ml. medium.

3. Carbohydrate source - appropriate amounts were added to give the required final carbohydrate concentration. Dextrin and starch were added before autoclaving but glucose, which was sterilized separately to avoid amino acid inactivation, was added afterwards. In the case of the cereals no additional carbohydrate was added since their native carbohydrate content was adequate (Rosen et al, 1962).

4. Distilled water - an amount of glass distilled water was added to bring the final medium volume to 10 ml. in each flask.

5. Sterilization - i) The flasks were then sterilized by autoclaving at  $121^{\circ}C$ . for 10 min. and allowed to cool to room temperature.

ii) Stock solution A (Appendix Table IV) diluted

10 x was cold sterilized in a Millipore filter unit with a  $0.20\mu$  plain membrane filter.

iii) Glucose solution (10% (w/v)) was autoclaved at 121<sup>0</sup>C. for 10 min.

6. Solution A - 1 ml 10 x diluted sterile solution A was added to each flask.

7. Glucose - If needed, an appropriate amount of glucose was added.

8. Inoculation - Each flask was inoculated with two drops (approximately 0.04 ml.) of a sample from the continuous culture apparatus of <u>Tetrahymena pyriformis</u> W using a disposable sterile hypodermic syringe (2½ ml.) and needle (18 G 1½).

9. Incubation - The culture flasks were incubated at room temperature in a <sup>3</sup>Gallenkamp automatic shaker operated at 60 oscillations/min. for specified periods.

10. Preservation of cells - After incubation the cultures were shaken on a flask shaker and 5 ml. of culture were transferred to a 20 ml. bottle containing 5 ml. of preservative fluid (90 ml. water, 20 ml. 36% (w/v) formaldehyde and 10 ml. of stock solution D). These preserved culture subsamples were stored at room temperature.

Growth Response

The growth response of Tetrahymena pyriformis W

3. Gallenkamp Water-Bath, Canlab, Toronto

was measured by either of two methods depending on the nature of the test protein:

# a) Microscopic Cell Counts:

The number of cells/ml. was determined microscopically using an improved Neubauer-ruled double chamber haemocytometer. The organisms on 9 sq. mm. areas of each haemocytomer chamber were counted. The mean of the 9 counts x 2 (dilution factor) gave the cell population x  $10^4$  per ml of the original culture. Before counting, all of the squares of each chamber were examined to determine that even filling had been obtained. Counting was repeated until a representative count was obtained. The growth responses of the organism to the cereal media were determined solely by this method since the presence of undigested fibrous material prevented use of the electronic counting method.

# b) Electronic Cell Counting and Sizing:

The <sup>4</sup>Coulter Counter Model B with a 200  $\mu$  aperture tube was used to determine cell numbers and mean cell volumes. The total population volume concentration (= mean cell volume x numbers/ml ) was used as a more accurate estimate of the growth response of <u>Tetrahymena</u> <u>pyriformis</u> W than cell numbers alone. This method of assessing the growth response was used for all the protein concentrates and the amino acid media since there were no large undigested particles in those cultures to affect

4. Coulter Electronics Inc., Hialeah, Florida

the accurate counting of cells or to plug up the aperture. The growth response of <u>Tetrahymena pyriformis</u> W to the cereals could have been assessed by electronic counting, only if the large undigested particles were elutriated from the cells in suspension without loss of cells. The elutriation procedure suggested by Teunisson (1971) was not employed since it did not seem practical for large numbers of samples.

The electrolyte used in the counting assembly and as a diluent consisted of 0.9% NaCl and 0.1% formaldehyde dissolved in distilled water, and it was filtered through a membrane of 0.4  $\mu$  porosity and counted to ensure a particle-free solution. The Coulter Counter was calibrated with ragweed pollen (20.195  $\mu$  diameter). The calibration constant (153  $\mu^3$ / threshold unit) relates particle volume to threshold units.

Four ml. aliquots of the preserved culture subsamples were diluted 1:20 with the electrolyte and 2 ml. were drawn through the Coulter Counter for counting and sizing of Tetrahymena pyriformis cells.

c) Growth Rate:

The growth rate of <u>Tetrahymena pyriformis</u> W in each medium was determined from a semi-logarithmic plot of  $\log_{10}$  (total ciliate volume concentration,  $\mu^3/ml$ .) versus time (hr.) The slope of the log phase multiplied by 3.3 corresponded to the exponential growth rate (hr.<sup>-1</sup>).

#### Respiration Rate

The effect of glucose, dextrin and starch on the respiration rate of resting cell suspension of <u>Tetrahymena</u> <u>pyriformis</u> W was determined by comparing the oxygen uptake using the conventional Warburg technique (Umbreit <u>et al</u>, 1964). The resting cell suspension was prepared by centrifuging 250 ml. of <u>Tetrahymena pyriformis</u> culture from the chemostat at a low speed and temperature (57 x G,  $2^{\circ}$ C.) for five minutes. The cells were washed twice in buffer (prepared from stock solutions B, C, and D) and finally concentrated 10 x by resuspending 25 ml. buffer.

Two ml. of the 24 hr.-starved cell suspension were pipetted into the ring of the Warburg flask. The centre well contained 0.2 ml. 2N NaOH wetting a piece of filter paper. The side arm was charged with 0.4 ml. of 10% (w/v) carbohydrate substrate, so that after mixing a final carbohydrate concentration of 1.25% was obtained. The oxygen uptake was measured at 10 min. intervals. For measuring endogenous respiration, the substrate was excluded and replaced by the same volume of buffer in the ring of the flask. For thermobarometer corrections the flasks contained only buffer, 3.2 ml. The experiment was conducted at 30<sup>0</sup>C. The initial cell density, 47 x 10<sup>4</sup> cells/ml. was determined using the Coulter Counter. Relative Nutritive Value

Protein quality was determined by comparing the

maximum growth response of the organism in the test protein medium to that in the standard protein, casein medium at the isonitrogenous level of 0.3 mg N/ml. Casein was assigned the value of 100 and the RNV's of test proteins were expressed as percentage of casein. The growth responses (mean cell volume, cell count, total volume) were recorded for different incubation periods and the maximum response (cell count or total volume) was used in calculating the RNV's. The carbohydrate source for RNV determination was soluble starch at 10 mg/ml. medium. In the case of the cereal media no carbohydrate was added.

#### Amino Acid Assay

The procedure for amino acid assays was based on the method of Stott, Smith and Rosen (1963) with  $5_{modifications}$ . Half of the total nitrogen content (ie. 0.15 mg N/ml.) of both the test protein medium and the standard amino acid mixture, (Solution F), was supplied by Solution F less the amino acid being assayed. The rationale for this modification was to ensure that the amino acids other than the one being assayed were in non-limiting concentrations. Since standard curves for such proteins as egg and casein revealed that the 0.3 mg N/ml. level was within the linear part of the response curve, it was decided to further standardize the assay by using this amount of nitrogen. The procedure

<sup>5.</sup> Private communication, Dr. E. B. Smith, Foods and Nutrition Department, University of Manitoba.

was similar to that used for RNV determination. The carbohydrate source was a (1:1) mixture of glucose and starch at 10mg /ml. medium. The amino acid content of the test medium was calculated according to the equation.



#### Amino Acid Supplementation

The effect of supplementing proteins with amino acids was tested following the procedure used for RNV determinations with the exception that the carbohydrate source was the glucose/starch mixture. Also an unsupplemented protein control was included. The levels of amino acid to be supplemented were calculated as percentages of 100 mg. protein.

## Statistical Analysis

The analysis of variance test (Snedecor and Cochran, 1968) of data in randomized block design was used to determine significant effects (P = 0.05).

EXPERIMENTAL AND RESULTS

#### EXPERIMENTAL AND RESULTS

# I a. The Effects of Type and Concentration of Carbohydrates on the Growth Characteristics of Tetrahymena pyriformis W

The effects of a monosaccharide, glucose, on the growth of <u>Tetrahymena pyriformis</u> W were compared to those of a polysaccharide, soluble starch. The comparison was made when the organism was supplied with three different nitrogen sources, the intact proteins, casein and whole egg, and an acid hydrolysate, Casamino Acids supplemented with 1% L - tryptophan all at isonitrogenous levels (0.3 mgN/ml.). The carbohydrate concentrations used were 2.5, 5.0, 10.0 and 20.0 mg/ml. medium. Also a control, containing no carbohydrate, was included for each nitrogen source tested.

The general assay procedure was followed and cultures were set up for each test medium for incubation times 18, 24, 36, 48, 60, 72, 96, and 120 hr. At the end of these incubation periods preserved subsamples of each culture were prepared and cell numbers, average cell volumes and total population volumes were determined from the data obtained using the Coulter Counter. In addition curves were drawn for  $\log_{10}$  total population volume,  $\mu^3/ml$ . vs time. The results of this study are shown in Tables 2 -4 and Figures 3 - 5.

#### Table 2

Mean Cell volume, maximum cell count, maximum population volume and growth rate of <u>Tetrahymena</u> pyriformis <u>W</u> in casein medium

with different concentrations of glucose and starch.

					the second s
mg/ml	Carbohy- drate	* Mean Cell Volume (x10 <sup>3</sup> u <sup>3</sup> )	** Maximum Cell Count (xl0 <sup>4</sup> /ml)	** Maximum Population Vol.(x10 <sup>9</sup> u <sup>3</sup> )	Growth Rate (hr <sup>-1</sup> )
0.0	Control	18.3	33.0	4.1	0.13
2.5	glucose .	23.0	68.4	9.7	0.14
	starch	25.9	68.6	9.7	0.16
5.0	glucose	29.1	74.7	11.0	0.16
	starch	32.9	72.3	9.9	0.16
10.0	glucose	29.4	76.3	14.4	0.16
	starch	29.0	78.3	13.9	0.15
20.0	glucose	33.0	62.9	14.2	0.13
	starch	29.5	59.7	12.3	0.15

\* These figures represent the highest of the mean cell volumes attained during the growth cycle.

\*\* The values for the maximum cell count and maximum total population volume are independent of each other since they were attained generally at different times during the growth of the population. Table 3.

Mean cell volume, maximum cell count, maximum population volume and growth rates of <u>Tetrahymena</u> pyriformis <u>W</u> whole egg protein medium (0.3mg N/ml) with different concentrations of glucose and starch.

			1. d.		·
mg/ml	Carbohy- drate	Mean Cell Volume (x10 <sup>3</sup> µ <sup>3</sup> )	** Maximum Cell Count (x104/ml)	** Maximum Population Vol.(x10 <sup>9</sup> µ <sup>3</sup> )	Growth Rate (hr-1)
0.0	Control	25.5	47.8	7.2	0.14
2.5	Glucose	30.3	99.4	14.6	0.18
	starch	24.6	98.6	15.4	0.17
5.0	glucose	28.1	94.7	15.0	0.21
	starch	31.5	109.6	16.1	0.23
10.0	glucose	31.0	84.4	15.7	0.20
	starch	34.2	108.5	19.6	0.18
20.0	glucose	35.2	68.8	19.8	0.17
	starch	30.0	90.4	19.0	0.18

\* These figures represent the highest of the mean cell volumes attained during the growth cycle.

\*\* The values for the maximum cell count and the maximum total population volume are independent of each other since they were attained generally at different times during the growth of the population.

#### Table 4.

Mean cell volume, maximum cell count, maximum population volume and growth rate of <u>Tetrahymena pyriformis</u>  $\underline{W}$  in casein hydrolysate medium with different concentations of glucose and starch

1			1 C	the sta	
mg/ml	Carbohy- drate	Mean Cell Volume ( 10 <sup>3</sup> µ <sup>3</sup> )	Maximum Cell Count (x10 /ml)	Maximum Population Vol.(x10 <sup>9</sup> µ <sup>3</sup> )	Growth Rate (hr <sup>-1</sup> )
0.0	Control	22.9	22.2	3.1	0.13
2.5	glucose	28.5	47.6	6.3	0.14
	starch	30.6	33.8	6.5	0.16
5.0	glucose	31.7	48.6	7.6	0.15
	starch	31.7	35.9	7.7	0.17
10.0	glucose	31.7	46.5	9.6	0.14
	starch	33.0	38.5	7.7	0.19
20.0	glucose	32.3	48.4	8.7	0.12
· · ·	starch	31.6	36.0	6.6	0.18

\* These figures represent the highest of the mean cell volumes attained during the growth cycle.

\*\* The values for the maximum cell count and the maximum total population volume are independent of each other since they were attained generally at different times during the growth of the population.

# A. Mean Cell Volume

The mean cell volume of <u>Tetrahymena pyriformis</u> grown in casein medium (Table 2) did not vary appreciably with the type of carbohydrate. However, a certain trend was noted: cells were consistently larger in starch at 2.5 and 5.0 mg/ml levels, of equal sizes at 10.0 mg/ml and larger in glucose at the high carbohydrate concentration 20.0 mg/ml. In the presence of carbohydrate the mean cell size increased up to 1.8 times that obtained in carbohydrate-free medium. Varying the carbohydrate concentration did not affect the mean cell volume at concentrations greater than 2.5 mg/ml; however at this low carbohydrate concentration the cells were much smaller.

In the whole egg protein medium (Table 3) glucose<sup>\*</sup> produced larger cells than starch at the lowest and highest carbohydrate concentrations. At 5.0 and 10.0 mg/ml levels the mean cell volumes were larger in the presence of starch than glucose. In addition the presence of carbohydrate in the medium increased the mean cell size up to 1.4 times than that in the control medium.

Varying the type and concentration of carbohydrate had no effect on the mean cell volume of <u>Tetrahymena</u> <u>pyriformis</u> grown in the acid hydrolysate of casein medium (Table 4). However, as was found for casein and the whole egg protein media, <u>Tetrahymena pyriformis</u> cells were larger in the presence of carbohydrate than in the carbohydrate-free medium.

A comparison of Tables 2, 3 and 4 revealed that the mean cell volume of <u>Tetrahymena pyriformis</u> W did not vary appreciably with the type of carbohydrate for concentrations greater than 2.5 mg/ml. Cells produced in either carbohydrate medium were larger than those in the control medium. Furthermore, for the three nitrogen sources being considered the mean cell size was not affected by the carbohydrate concentration.

# B. Maximum Cell Count

Higher cell numbers were attained in the starch media at all carbohydrate concentrations greater than 2.5 mg/ml when whole egg protein was the nitrogen source. On the other hand in the casein hydrolysate medium higher cell numbers were produced in the presence of glucose than starch at corresponding carbohydrate concentrations. In the casein medium the maximum cell numbers were approximately equal in the presence of either carbohydrate at similar concentrations. Thus the effect of varying the type and concentration of carbohydrate on the maximum cell count seemed to depend on the nitrogen source in the growth medium.

# C. Maximum Total Population Volume

The maximum total population volume (total population volume = cell numbers x mean cell volume), an estimate of biomass, was greater in the presence of glucose than

corresponding concentrations of starch at carbohydrate levels greater than 5.0 mg/ml for the casein hydrolysate medium. In the whole egg protein and casein media there were not many differences in the total population volumes between corresponding concentrations of glucose and starch. Generally, increasing the concentration of glucose and starch from 2.5 mg/ml to 20.0 mg/ml , produced increased maximum growth responses up to a saturation point.

#### D. Growth Curves and Growth Rates

The growth curves  $(\log_{10} \text{ population volume vs time})$  describing the growth of <u>Tetrahymena pyriformis</u> W in varying concentrations of glucose and starch with different nitrogen media are shown in Figures 3, 4 and 5. The growth rates are summarized in Tables 2, 3, and 4.

In the casein medium, the presence of different concentrations of either glucose or starch did not affect the growth rate appreciably. The lowest and highest glucose levels 2.5 and 20.0 mg/ml resulted in lower growth rates and the high glucose concentration (20.0 mg/ml ) repressed the growth of the organism. This repression of growth was manifested by a concomitant increase in the lag phase and a decrease in the growth rate. In spite of this, the maximum growth response in the presence of the high glucose concentration was comparable to that in the presence of the other carbohydrates.

Figure 3: Growth of <u>Tetrahymena</u> pyriformis <u>W</u> in Casein medium (0.3mgN/ml) with different carbohydrates at varying concentrations.



TIME (Hrs.)

Figure 4: Growth of <u>Tetrahymena pyriformis</u> W in whole egg medium (0.3mgN/ml) with different carbohydrates at varying concentrations.



Figure 5: Growth of <u>Tetrahymena pyriformis W</u> on casein hydrolysate medium (0.3mgN/ml) with different carbohyrates at varying concentrations



The duration of the exponential growth phase varied with the types of energy and nitrogen sources. The concentration of carbohydrates did not show any effect on the logarithmic growth phase, except that the log phase was longer in high concentrations of glucose (20.0 mg.ml). In the casein medium the log phase lasted 72 hr for all carbohydrate concentrations except glucose at 20.0 mg/ml which lasted for approximately 96 hr. Shorter log phases were observed for the whole egg protein medium, 72 hr in the presence of 20.0 mg/ml glucose and 60 hr for the other concentrations of either carbohydrate medium In casein hydrolysate the log phase was 72 hr for starch, 84 hr for 2.5 mg/ml glucose.

Higher growth rates were observed for the polysaccharide medium than the monosaccharide medium at concentrations greater than 5.0 mg/ml. The mean growth rate of <u>Tetrahymena pyriformis</u> W in casein hydrolysate medium in the presence of starch was 0.18 hr<sup>-1</sup> compared to 0.14 hr<sup>-1</sup> for glucose. The mean growth rates in either glucose or starch medium were 0.16 hr<sup>-1</sup> for casein and 0.18 hr<sup>-1</sup> for the whole egg protein medium.

The results of this investigation, of the growth responses of <u>Tetrahymena pyriformis</u> W to different concentrations of the monosaccharide, glucose, and the polysaccharide, soluble starch, in media containing different nitrogen sources, were analyzed statistically.

The analysis of variance test was applied to the

data from Tables 2, 3 and 4 and the results are recorded in Appendix Tables vi, vii, viii and ix and summarized in Appendix Table xiv. These results revealed that four of the variables were statistically significant (P = 0.05). First, the protein source (P) significantly affected the mean cell volume, the maximum cell count, the maximum total volume and the growth rate of Tetrahymena pyriformis W, second, the carbohydrate concentration or level (L) produced significant effects on the mean cell volume, the maximum cell count and the maximum total volume. Third, the interaction between the protein source and the carbohydrate level (PL) was significant on the maximum cell count and the maximum total volume. Finally, there was a significant interaction between the protein source and the carbohydrate type (PT) on the maximum cell count. The significant interaction effects (PT and PL) are illustrated in Appendix Figures ii, iii and iv.

Ιb.

# Effects of Equal Concentrations of Glucose, Dextrin, Soluble Starch and a Glucose/Starch Mixture on the Growth of <u>Tetrahymena pyriformis</u> W on the Intact Protein <u>Medium and a Crystalline Amino Acid Mixture Medium</u>

Glucose, dextrin, soluble starch and a mixture (1:1) of glucose and starch at one level 10.0 mg/ml medium were compared as energy sources for <u>Tetrahymena pyriformis</u> W when the nitrogen source was either the intact protein, whole egg or the free amino acid mixture, solution F, at isonitrogenous levels 0.3 mgN/ml medium.

The experimental procedure was similar to that outlined for the previous study. Growth responses were determined using the Coulter Counter for incubation periods 18 hr to 120 hr for each test medium. The maximum cell count, maximum total volume, mean cell volume and the growth rate are indicated in Table 5. Also the growth curves obtained by plotting  $\log_{10}$  total population volume versus incubation time are shown in figures 6 and 7.

In the intact protein medium, the maximum number of cells produced using dextrin, starch and glucose/starch as energy sources were similar but up to 1.3 times higher than that using the same concentration of glucose. On the basis of total population volume the growth responses of <u>Tetrahymena pyriformis</u> W in the presence of starch and glucose/starch were similar but higher than those in the presence of glucose and dextrin. The observation, that dextrin produced a higher maximum cell count than glucose in the whole egg protein medium, but the same maximum total

(10.0mg/ml) on the growth	(intact vs free amino acid)
Table 5 Effects of different carbohydrate sources at one concentration	responses of <u>Tetrahymena pyriformis W</u> grown in different forms

of nitrogen media

Carbohydrate 10mg/ml	Mean Ce (x 10 <sup>3</sup> ,	ell Volume /μ3) *	Maximun (x 10 <sup>4</sup> /	n Cell Count (ml) **	Maximum Volume	Population (x $109\mu^3$ ) **	Growth <sup>1</sup> (hr <sup>-1</sup> )	Rate
· · · · · · · · · · · · · · · · · · ·	Egg	Soln. F	Еgg	Soln• F	Egg	Soln• F	Egg	Sol <sup>n</sup> • F
Control (none)	17.5	22.2	47.8	15.8	7.3	4.2	0.14	0.16
Glucose	35.7	31.1	84.4	58.1	15.7	11.2	0.20	0.21
Dextrin	33.2	35.1	103.4	56.0	15.9	11.8	0.23	0.20
Starch	37.4	36.3	108.5	30.4	19.6	8.6	0.19	0.17
Glucose/ starch	36.2	34.2	109.6	61.6	19.5	11.8	0.20	0.18
* These figure	s repres	ent the high	lest of th	le mean cell	volumes a	ttained durin	g the grow.	th cycle.

of each other since they were attained generally at different times during the growth of the population The values for the maximum cell count and the maximum total population volume are independent \* \*

Figure 6: Growth of <u>Tetrahymena pyriformis</u> <u>W</u> in whole egg medium (0.3mgN/ml) with different carbohydrates at 10.0 mg/ml concentration



Figure 7: Growth of <u>Tetrahymena pyriformis W</u> in Sol<sup>n</sup> F (crystalline amino acid mixture, 0.3mgN/ml) with different carbohydrate sources at 10.0 mg/ml concentrate


population volume, could be explained by the smaller sized cells in the presence of glucose.

The effects of the four carbohydrate sources on the growth of the organism in the free amino acid medium, solution F were different from those observed in the intact protein medium. The maximum growth response (cell count and total volume) in soluble starch was lower than in glucose, dextrin or glucose/starch. The growth rates did not vary with the carbohydrate source for either the intact protein or the free amino and medium. Also both forms of nitrogen sources gave similar growth rates of the organism.

Thus, this study comparing the effects of different types of carbohydrate sources at a uniform concentration on the growth of <u>Tetrahymena pyriformis</u> W when the nitrogen was supplied by either the intact protein, whole egg or the free amino acid medium, solution F, showed that:

i) The mean cell volume and maximum cell count did not vary either with different carbohydrate or nitrogen sources.

ii) Dextrin, starch and glucose/starch stimulated a higher maximum cell count than glucose in the whole egg protein medium. Dextrin, glucose and glucose/starch stimulated higher maximum cell counts than starch - the amino acid mixture medium.

iii) Starch and glucose/starch produced higher
maximum total population volumes in whole egg protein
medium; whereas glucose and glucose/starch produced a

higher polulation volume in solution F.

From these observations it was apparent that the carbohydrate source which produced the optimal maximum growth response in both the whole egg intact protein medium and the free amino acid mixture medium was the 1:1 mixture of glucose and starch.

The analysis of variance test (Appendix Tables  $\bar{x} - xiii$ ) of the data from this study revealed that the form of the nitrogen source (intact protein vs gree amino acids) and the type of carbohydrate (glucose, starch, dextrin, glucose/starch) significantly affected only the maximum total volume and the growth rate. The protein - carbohydrate type (PT) interaction produced a significant effect on the maximum total volume.

II The Effects of Extracellular Carbohydrates on the Respiration of Tetrahymena pyriformis W

Table 6 illustrates the effect of 12.5 mg/ml glucose, dextrin and soluble starch on the endogenous respiration of <u>Tetrahymena pyriformis W cells</u>.

The endogenous respiration rate,  $4.12 \times 10^{-5} \mu l$  $0_2/hr/cell$  was stimulated barely by glucose (11%) and more highly by dextrin (32%). The presence of starch, however, decreased this endogenous rate by 38%. Table 6. Effect of Extracellular <sup>C</sup>arbohydrates on Respiration. Respiration of <u>Tetrahymena pyriformis W</u> followed for 70 minutes. Table gives 0<sub>2</sub> uptakes. Total volume 3.2ml; temp. 30°C; gas phase air; Initial celldensity 47x10<sup>4</sup>/ml.

Substrate added	Respiration Rate $\mu^1  0_2 / hr/cell$	% Stimulation	فاستعادهم والقارب والأباسين لأستر والمناجر المراجع والسيورية
Endogenous	4.12 x 10.5		
Glucose	4.56 x 10 <sup>.5</sup>	11	وفيتوجه والقنيم والاختبارين أدحه متموا ويسبب أيتريهم والمريسية المريسية
Dextrin	5.44 x 10 <sup>.5</sup>	32	ووجارتها والمحافظة والمتعادية والمحافظة والمحافظة والمحافية والمحافية والمحافية والمحافية
Starch	2.57 x 10 <sup>.5</sup>	-38	

50

### IIIa. Evaluation of Protein Quality Using Tetrahymena Pyriformis W

Test proteins were evaluated relative to a standard or reference protein, casein. The media contained the protein at 0.3 mgN/ml and 10 mg/ml soluble starch except in the case of the cereals which did not require an additional carbohydrate source. Each test included the reference protein to ensure that the relative growth responses were assessed under similar conditions. Duplicate cultures of each protein were incubated for 12 hr intervals up to 120 hr. The maximum growth responses were used in the determination of the relative nutritive values (RNV) of the test proteins.

Table 7 shows the RNVs of the protein concentrations of whole egg, casein, soybean, cod frames, herring, faba bean, zein, the faba bean isolate (natural vegetable protein, NVP); the acid hydrolysate of casein (Casamino Acids) and the free amino acid mixture, solution F. The mean cell volume, 96 hr cell count and maximum total population volume with the corresponding RNVs are given for each nitrogen source.

It was noted that there was considerable variation in the mean cell volumes of <u>Tetrahymena pyriformis</u> grown in the different protein media from 11.7 x  $10^3 \mu^3$  for zein to 33.4 x  $10^3 \mu^3$  for the whole egg protein medium compared to 24.7 x  $10^3 \mu^3$  for the standard protein, casein. This wide variation suggested the importance of including the mean cell volumes in any equation for the determination of

grown on different nitrogen sources (0.3mgN/ml) and their corresponding relative nutritive values Average Cell volume, 96<sup>h</sup> cell count, maximum total population volume of Tetrahymena pyriformis <u>W</u> Table 7.

	Average	96h Cell	Count	Max. popl <sup>r</sup>	<sup>1</sup> Volume	Literature Ve	alues	
Nitrogen source	$\operatorname{ume} (10^{3}\mu^{3})$	(x 10 <sup>4</sup> /m]	0\0	x109u3/ml	RNV	T. pyriformis	Rat	
•			Саѕели			RNV	NPU % of Casein	Protein Index (e)
Casein	24.7	54	TOO	14.5	100	100	.00.	2.52
Egg, whole	33.4	68	165	19.5	135	a, b c 124,128 * c f b	u c 137,134	
soy, Conc.	29.3	60	111 <sup>.</sup>	15.3	106	74,87,107	106,80	2.12
Faba, conc.	31.1	. 29	54.	8.6	59			0.26
Faba, isolate	27.0	38	70	8.1	56	ţ		
(NVP) Herring, conc.	21.7	18	33	5,9	41	таа 78,130,93		
Cod, frames	23.2	3.5	65	7.7	53	00T**		
Zein	11.7	Ϋ́	9	0.4	m	18		
Casamino acid	30.5	38	58	۲ ۴ 6	67			
Solution F	32.61	48	73	12.0	83			
		-						1
a Rosen and Feri	nell (1956	e P	McDonald an	nd Larter (197	4)			5

\*\* muscle

\* meal

Helms and Rolle (1968)

44

Block and Mitchell (1946-47)

Kamath and Ambegaokar (1968)

υ יס

Baum and Haenel (1965)

൧

relative protein quality involving growth responses of <u>Tetrahymena pyriformis</u> W. Thus the rankings of the protein (Table 7) based on the 96 hr cell counts differed in certain cases from those based on the maximum total population volume (estimate of biomass). The effect of using either cell count or total volume on the RNVs of the proteins was observed especially for cod (RNV <sub>count</sub> = 65, RNV<sub>volume</sub> = 53), faba bean isolate, nvp (RNV <sub>count</sub> = 70, RNV<sub>volume</sub> = 56) and casein hydrolysate (RNV <sub>count</sub> = 58, RNV<sub>volume</sub> = 67).

The RNVs based on total population volume gave a more valid assessment of protein quality since it included the cell volume differences. These RNVs indicated that relative to casein (RNV = 100), the whole egg protein (RNV = 135) and soy protein concentrate (RNV = 156) were the highest quality proteins. The crystalline amino acid mixture (RNV = 83), which simulated the amino acid requirements of the organism produced a higher growth response than the tryptophansupplemented Casamino Acids. It was noteworthy that the effect of acid hydrolysis significantly reduced the nutritive value of the intact protein, casein. Both faba bean samples, viz. the concentrate (RNV = 59) and the isolate, NVP (RNV = 56) were evaluated as similar quality proteins. However, the seemingly-underestimated nutritive values for cod (RNV = 53) and herring (RNV = 41) together with that of zein (RNV = 3) might have been due partly to the low solubilities of these proteins.

The RNVs for the CIMMYT cereals, wheat and triticale

and the Protein Index (PI) determined by 14 day rat-feeding experiments are given in Table 8. The <u>Tetrahymena</u> evaluation of the cereals ranked the three triticale cereal proteins superior in quality to the wheat samples. Also the results indicated that the organism was capable of differentiating between the two CIMMYT wheat proteins (4859, RNV = 48, 4860 RNV = 53) and the three types of triticale (4861 RNV = 63, 4862 RNV = 75, 4863 RNV = 74). The <u>Tetrahymena</u> nutritive values correlated highly with the rat PI values (r = 0.96).

CIMMYT CEREAL	Tetrahymena RN <b>V *</b> *	Rat PI *
4859-7-Cerros-Wheat	48	1.45
4860-Inia-Wheat	53	1.49
4861-PM-132 Triticale	63	1.78
4862-PM-2-Triticale	75	1.88
4863-PM-15-Triticale	74	1.82
Casein	100	2.50

## Table 8. Relative Nutritive Values of CIMMYT cereals using

Tetrahymena pyriformis W

RNV/PI: Coefficient of variation r = 0.96

PI = 0.02 RNV + 0.43

\*Protein Index (PI) = gms body weight gain gms protein consumed during 14 day test periods on 10% protein diets.

from McDonald and Larter (1974)

\*\* calculated using maximum cell counts.

Available Lysine, Threonine and Methionine plus cystine contents for CIMMYT cereals Table 9.

using Tetrahymena pyriformis  $\underline{W}$  (mg/l00mg protein)

•									
CIMMYT	Lys:	ine		Three	onine		Meth	ionine & Cy:	stine
CEREAL	Tetra- hymena	Chemical	Avail- ability	Tetra- hymena	Chemical	Avail- ability	Tetra- hymena	Chemical	Avail- ability
4859-Wheat	2°2	3°-1	81%	с. Т	3.1	42%	l.8	8°. 3	47%
4860-Wheat	2°2	3° J 3	81% 8	1.5	3.2	478	2.1	4 ° 1	51%
4861-Trit- icale	2.9	ي ۳	% M 0%	2.0	3 ° 0	67%	й. С	3.1	110%
4862-Trit- icale	3°0 3	7°8	% 00 00	2.5	a. N	78%	3.2	3.7	87%
4863-Trit- icale	3°0	3°4 3	% 00 00	2.2	0 	73%	2.0	б <b>°</b> е	51%
					and a second and a s				56

\* Chemical values - private communication from Dr. Mertz Biochemistry Dept., Purdue

University.

### III b. Amino Acid Assays

Cultures of <u>Tetrahymena pyriformis</u> W containing  $\begin{bmatrix} (Test protein) + (Sol<sup>n</sup>F - amino acid being assayed) \\ (0.15 mgN/ml) & (0.15 mgN/ml) \end{bmatrix}$  and  $\begin{bmatrix} (Sol<sup>n</sup>F) \\ (0.15 mgN/ml) + (F - amino acid being assayed) \\ (0.15 mgN/ml) \end{bmatrix}$  were compared in duplicate for 60 hr, 72 hr and 96 hr. The cultures supporting maximum growth responses were used to determine the available amino acid content of the test protein. Proteins assayed were the CIMMYT cereals for lysine, isoleucine, threonine and tryptophan.

The results of the <u>Tetrahymena pyriformis</u> assays on the CIMMYT cereals for lysine, threonine and methionine plus cystine are shown in Table 9 together with the chemically determined values. The microbilogically determined lysine content of the cereals varied from 2.5 mg/100mg protein for the wheat samples to 3.0/100mg protein for the triticale sample. According to these results lysine seemed to be readily available (81-88%) to the organism. A statistical analysis of the values in Tables 8 and 9 for the CIMMYT cereals revealed the following correlations and regressions:

a) between the <u>Tetrahymena</u> available lysine values (X) and the chemically determined lysine values (Y) Y = 0.67X + 1.4 (r = 0.92),

b) between the <u>Tetrahymena</u> available lysine values (X) and the PER values (Y) Y = 0.90X + 0.82 (r = 1.0),

c) between the <u>Tetrahymena</u> available lysine (X) and the RNVs (Y) Y = 45X + 62.5 (r = 0.96).

The availabilities of threonine in the cereals to <u>Tetrahymena</u> served to differentiate clearly between the wheat and triticale. Low threonine availabilities of 42 and 47% were obtained for the wheat samples compared to 67, 78 and 73% availabilities for the three triticale proteins. An interesting correlation existed between the available threonine content of the CIMMYT cereals (X) and the corresponding relative nutritive values (Y):Y = 24.0X + 17.0 (r = 0.98).

The results of the methionine plus cystine assay of the CIMMYT cereals showed low availabilities for both wheat 4859 and 4860 samples and triticale 4863 and significantly higher availabilities for 4861 and 4862 triticale samples.

The available isoleucine, lysine, threonine and tryptophan contents of casein, whole egg and faba bean concentrate as assessed by <u>Tetrahymena</u> are summarized in Table 10. Generally it was observed that, of the four amino acids assayed by <u>Tetrahymena</u> in these test proteins, isoleucine was the most available and lysine the least available. Also the availabilities of threonine for casein (54%) and whole egg protein (57%) were found to be lower than that of faba bean concentrate (76%). The values for tryptophan agreed reasonably well with the literature values for the three test proteins. In addition, the high availabilities of tryptophan for these proteins were noteworthy.

Available isoleucine, lysine, threonine and tryptophan contents (mg/100 mg protein) of casein, whole egg faba bean concentrates using Tetrahymena pyriformis <u>W</u>. Table 10.

		-										
ſ	Isole	ucine		Lysine	41		Threoni	ne		Trypto]	phan	
teins .	Tetra- hymena	* Chem- ical	% avail- ability	retra- hymena	Chem- ical	% avail- ability	Tetra- ĥymena	Chem- ical	% avail- ability	retra- hymena	Chem- ical	% avail- ability
Casein	4 8	ى ب	ون ح ص	4. 7	ი დ	% 23 2	2.6	4 <b>.</b> 8	70 %	0.97	* * * 	% 99
ភ្លេថ	4.6	e . 3	73%		7.0	% 0 2)	0°0	5.1	57%	1.1	. 4*. * * 4	79%
Faba Bean	5.4	0.5	- 80 C T	0. V	0	% 17 Q	5.6	Ф. С	76%	0.92	8 0 0	107%

\* FAO (1970)

Colormetric determination obtained from Porter and Rolls (1973) \* \*

\*\*\* Microbiological literature value from FAO (1970)

### III c. Amino Acid Supplementation

The effects of supplementing protein sources with amino acids on the growth response of Tetrahymena and on the nutritive value of the protein were investigated. Faba bean protein concentrate was supplemented with the most limiting amino acid methionine at 0.125%, 0.375%, 0.750% and 1.0% of the protein. The carbohydrate source was supplied by glucose and starch (1:1) at 10 mg/ml. The experiments were conducted with the supplemented protein, an unsupplemented control and the reference protein, casein. Duplicate cultures were incubated at 72 hr and 96 hr and the maximum cell population volumes were used to determine RNVs. The effects of supplementing faba bean concentrate with methionine are shown in figure 8 and Table 11. The addition of methionine to the faba bean protein improved the nutritive value and at 0.375% concentration the protein quality was comparable to that of the reference protein, casein.

A similar experiment was conducted to determine possible effects of supplementing both fish protein samples, cod frames and herring FPC with tryptophan. In this case (Table 12) no improvement in the protein quality was observed and in addition 1.12% L - tryptophan reduced the nutritive value of cod.

The effects of the supplementation of the wheat and triticale CIMMYT cereals with lysine and methionine were also studied. Each of the five CIMMYT cereals were

Figure 8: Effect of DL-methionine (met) supplementation on the relative nutritive value of Faba bean concentrate using <u>Tetrahymena pyriformis W</u>



## Table 11. The effect of DL-methionine supplementation of the RNV of faba bean concentrate determined by <u>Tetrahymena pyriformis W</u>

Protein	Maximum Total Vol. (x 10 <sup>9</sup> µ <sup>3</sup> )	RNV
Casein	13.8	100
Faba bean (control)	7.9	57
Faba bean + 0.125% met.	10.4	75
Faba bean + 0.375% met.	12.8	93
Faba bean + 0.750% met.	15.0	109
Faba bean + 1% met.	16.6	120

## Table 12: The effects of L-tryptophan supplementation on the RNV's of cod and herring protein concentrates using <u>Tetrahymena pyriformis W</u>

▲ - \*\*

Protein	Maximum Total Volume(x 10 <sup>9</sup> µ <sup>3</sup> )	RNV
Casein	13.8	100
Cod (control)	7.3	53
Cod + 0.56% trp	7.6	55
Cod + 1.12% trp	5.1	37
Herring (Control)	6.6	48
Herring + 0.56% trp	6.6	48
Herring + 1.12% trp	6.2	45

supplemented with 1.7% (w/w) lysine in the form of L lysine HCl and 0.6 and 1.2% DL - methionine, singly and in combination. The results summarized in Table 13 and figure 9 showed that methionine supplementation depressed the growth response of Tetrahymena pyriformis W. This inhibitory effect of methionine became more evident when the concentration of methionine was increased to 1.2% and with the relatively inferior quality cereals (4859 wheat, 4860 wheat and 4861 triticale). Lysine supplementation at the one level used did not affect the nutritive value of these three CIMMYT cereals (4859, 4860 and 4861). However, lysine supplementation improved the nutritive value of the two superior quality triticale samples (4862 and 4863) to that comparable with or even better than the reference protein, casein. Supplementation of the cereals with the combination of 1.7% lysine and 0.6% methionine stimulated a higher growth response in all cases except for the lowest quality cereal, 4859 wheat. The improvement in protein quality seemed to increase with the quality of the unsupplemented cereal. Even though the addition of a higher supplemental level of 1.2% methionine together with 1.7% lysine did not stimulate a further increase in the growth response compared to lower supplemental level of methionine plus lysine, it did improve the nutritive value of the unsupplemented triticales 4862 and 4863.

Table 13. Effects of Supplementation of the CIMMYT cereals with L-lysine HCl and DL-methionine on their relative nutritive values using <u>Tetryahymena pyriformis W</u>

	Relati	ve Nutri	tive Value:	s of CIMM	NYT Cerals
Treatment	4859 -7-Cerros Wheat	4860 Inia- Wheat	4861 PM-132- Trit.	4862 PM-2- Trit.	4863 PM-15- Trit.
Control (unspple- mented	48	53	63	75	74
Cereal + 1.7%lys	41	51	68	101	113
Cereal + 0.6% met	37	33	60	66	68
Cereal + 1.2% met	18	22	40	58	66
Cereal + 1.7% lys and 0.6% met	25	59	90	110	96
Cereal + 1.7% lys and 1.2% met	18	41	62	95	83

65

Figure 9: Effects of L-lysine hydrochloride and DL-methionine supplementation on the Relative Nutritive Values of CIMMYT wheat and triticale using <u>Tetrahymena</u> <u>pyriformis W</u>



DISCUSSION

#### DISCUSSION

#### Part I

a. The effects of varying the type and concentration of carbohydrate on the growth response of <u>Tetrahymena</u> pyriformis to different protein media.

b. The effects of varying the type of carbohydrate at 10.0 mg/ml concentration on the growth response of <u>Tetrahymena pyriformis</u> W to an intact protein and free amino acid media.

The statistical analyses of Part I are summarized in Appendix Table xiv. The mean cell size, maximum cell count, maximum total population volume and growth rate were used as indices of the growth response of <u>Tetrahymena</u> <u>pyriformis</u> W. In Part I a. the protein source, carbohydrate level (concentration), and the protein/carbohydrate type and protein/carbohydrate level interactions produced effects on the growth response of the organism. In addition the analysis of variance of Part I b. revealed that, when comparing an intact protein and free amino acid media, the type of carbohydrate had significant effects on the maximum total population volume and the growth rate of Tetrahymena pyriformis W.

Considering the different growth media certain consistent trends were noted. Of the intact protein media (Tables 2 and 3) casein stimulated similar maximum cell counts and total volumes in the presence of either glucose or starch but whole egg protein produced greater maximum cell counts in the presence of starch than glucose at all concentrations greater than 2.5 mg/ml. In the case of the protein hydrolysate medium (Table 4), the monosaccharide stimulated higher cell counts (at all levels) and greater population volumes (at levels greater than 5.0 mg/ml ). In addition it was found that the simultaneous presence of glucose and starch duplicated the optimal growth response produced by glucose in the amino acid medium and by starch in the intace whole egg[protein medium (Table 5).

These results agree partly with those of Reddy (1965) and Estevez (1962) who reported that glucose, in the amino acid medium stimulated greater growth responses than either dextrin or starch and in the intact casein medium, was as equally growth-promoting as the polysaccharides. However, these results disagree with the findings of Stott et al (1963) and Reddy (1965) that glucose was a better growthpromoting carbohydrate than the polysaccharides for intact proteins other than casein. There is also disagreement with the results of Reynolds and Wragg (1962) who found that the polysaccharide, dextrin was a better energy source than the monosaccharide, glucose in the amino acid medium. These apparent diversities in results are not unexpected when one considers that different methods were used for measuring growth response and that the data from those studies were not analyzed statistically. The optical density measurements used by Estevez (1962) and Reynolds

and Wragg (1962) have proven useless according to Levy and Wasmuth (1970) mainly because of the effect of the highly variable glycogen content of the Tetrahymena cells. The colorimetric method used by Reddy (1965), employing the enzymatic reduction of 2, 3, 5 - triphenyltetrazolium chloride, has been rejected on the basis of inaccuracies (Jambor, 1955; Rosen and Fernell, 1956). The drawback of estimating the growth response of the organism by cell numbers only, as was done by many workers (Stott et al, 1963; Kamath and Ambegoakar, 1968; Helms and Rolle, 1968) is illustrated by the research reported in this study (figure 10). Cell volumes do not increase in parallel with cell numbers. Thus the product of the mean cell volume and the cell count (= total population volume) would be a more accurate index of the entire growth response and it could be expected to yield different results from the cell count only measurements.

Reynolds and Wragg (1962) and Reynolds (1964) reported that dextrin was a superior growth stimulator than glucose in the defined medium. In attempting to explain this difference, Reynolds (1970) found a two-fold increase in the total of three non-essential amino acids (alanine, glycine and glutamic acid) which were sythesised when glucose replaced dextrin in the defined growth medium. He therefore concluded that an increased diversion of essential to non-essential amino acids by <u>Tetrahymena</u> when required to metabolize glucose must be an immediate

Figure 10: Charges in cell numbers, mean cell size and total population volume of <u>Tetrahymena</u> <u>pyriformis</u> <u>W</u> grown in whole egg protein medium containing 10.0 mg/ml dextrin as a function of time



cause of parallel loss in efficiency of nitrogen utilization as compared to those cells provided with the polysaccharide. This author questions the validity of such a conclusion for the following reasons:

 There was no direct proof that the nonessential amino acids "synthesized" were derived from the essential amino acids in the medium.

2) The lack of information on a control carbohydrate-free medium.

3) The fact that the polysaccharide is eventually metabolized in a similar manner as glucose.

Other possible explanations for the differences in growth responses of Tetrahymena pyriformis to different types of carbohydrates and different forms of nitrogen sources (intact protein vs free amino acids) include: i) osmotic effects; ii) stimulatory effects of particles of intact protein in the growth medium; iii) differences in the rates of availability of amino acids and glucose for protein synthesis. The results of Reynolds and Wragg (1962) and the higher growth response, observed in this study, with the medium of higher osmotic pressure (solution F + glucose, Table 5) compared to the medium of lower osmotic pressure (solution F + starch) would appear to eliminate the importance of the osmotic effect hypothesis. Rasmussen (1973) reported increased growth of Tetrahymena pyriformis due to mechanical stimulation of food vacuole formation induced by the presence of particulate matter

in the medium. This could account for some of the difference observed in the growth response of the organism to the intact protein medium and the amino acid solution (Table 5). The higher growth response of the organism in casein medium compared to the casein hydrolysate medium (Tables 2 and 4) could also be explained partly by the stimulatory effect of particulate matter. The production and characterization of an extracellular a - amylase (Smith, 1961) and protease (Viswanatha, 1956; Smith, 1961) of Tetrahymena have been studied. There is, however, some controversy as to the exact nature of nutrient uptake: large polymer particles such as of protein and polysaccharide are ingested intact into food vacuoles to be hydrolyzed or they are hydrolyzed partly or fully (Muller and Rohlich, 1961) outside of the cell. Nonetheless, for efficient nitrogen utilization or protein synthesis the amino acids and glucose should be available simultaneously. If the amino acids were made available to the organism much before glucose then they would be utilized "wastefully" for energy production resulting in a decreased growth response. Thus the reason for a greater growth response in the amino acid medium containing glucose (Table 5), compared to that containing starch, could be utilization of the amino acids for energy production and protein synthesis during the time taken for the uptake and hydrolysis of starch to Similarly the reduced growth response of glucose. Tetrahymena pyriformis in the amino acid medium (Table 5)

containing starch compared to that containing dextrin could be due to differences in the rates of conversion of starch and dextrin to glucose (Smith, 1961). The observation that the simultaneous presence of glucose and starch in the amino acid medium (Table 5) produced a growth response greater than that produced in the same medium containing starch but equivalent to that in the presence of glucose supports the third hypothesis. Similar explanations could account for the higher growth response of the organism in the whole egg protein medium containing starch or dextrin compared to that containing glucose (Table 5). In the polysaccharide medium similar rates of availability of glucose and amino acids produces an optimal growth response. On the other hand, in the monosaccharide medium, the amino acids resulting from hydrolysis of the intact protein became available after the glucose had been stored in the cells. Thus the amino acids could have been utilized initially for energy production and protein synthesis during the time required for glucose to be made available again.

Growth repression caused by high levels of glucose has been observed by other workers (Estevez, 1962; Reynolds and Wragg, 1962; Fernell and Rosen, 1956). It was noted in the results (figures 3, 4, and 5) that the respression of the growth of <u>Tetrahymena pyriformis</u> by 20.0 mg/ml (2% w/v) glucose was manifested only as an increase in the lag phase and not as a decreased maximum

growth response. It is noteworthy that a decreased growth response at this concentration reported by the other workers could be due to the fact that in those cases growth was measured only at one point of the growth curve, presumably prior to the establishment of the maximum phase. This prolonged lag phase caused by high concentrations of glucose is probably the result of glucose repression of protein biosynthesis. As a matter of fact it is known that a number of inducible enzymes in microbial systems are either not synthesized or else are synthesized at reduced rates in the presence of high concentrations of glucose. In particular Dickie and Liener (1962) have reported on the repression by glucose of the proteolytic activity of <u>Tetrahymena</u> pyriformis.

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Reynolds and Wragg (1962) using the rate of carbohydrate disappearance from the growth medium as a measure of its utilization concluded that the polysaccharide, dextrin was utilized more rapidly than the monosaccharide, glucose, in defined media. However, other evidence in the literature that in <u>Tetrahymena pyriformis</u> glucose is converted to glycogen (Hogg <u>et al</u>, 1956; Levy and Wasmuth, 1970) would invalidate the use of carbohydrate uptake as an index of its utilization.

# Part II The Effects of Extracellular Carbohydrates on the Respiration of Tetrahymena pyriformis W

The results of the metabolic experiment (Table 6) are most interesting. The relatively high endogenous respiration rate compared to that in the presence of the carbohydrates indicate that the organism was utilizing intracellular compounds for energy production. The marginal stimulation by glucose (11%) confirmed the results of Ryley (1952). However, the depression of the endogenous respiration rate by starch, cannot be explained. These results coupled with those of Ryley (1952): RQ endogenous = 0.63; RQ glucose = 0.48, suggest that intracellular lipids or nitrogenous compounds may play a significant role in energy production in Tetrahymena. The nature of the medium used to grow the organism might have influenced these results. Τt has been shown (Conner and Cline, 1967) that cells grown in the presence of glucose, utilize it very well, as judged by the increase in respiratory rate, whereas those grown without glucose require supplements of sodium and potassium ions to show an equivalent increase in respiratory activity. The cells used in these experiments were grown in a glucose-free proteose peptone medium.

The work of Dewey and Kidder (1972) represents an important contribution to the understanding of the relative roles of carbohydrate and amino acids in the energy production in <u>Tetrahymena</u>. Using <sup>14</sup>C-labelled

substrates it was shown that the amino acid proline was oxidized more rapidly and earlier in growth than glucose. The low rate of glucose oxidation was thought to be due to the large extent to which it is converted to cellular components such as alanine, aspartate and glutamate. Thus stimulation of respiration by amino acids could be considerable in comparison to glucose and it was suggested that amino acids are degraded largely for obtaining energy, even in the presence of other carbon compounds such as acetate and glucose. Proline in particular served as a readily available energy source being converted rapidly and completely to glutamate. Thus it is becoming more evident that amino acids instead of carbohydrates might be primary energy sources in Tetrahymena pyriformis. Ιt would be most interesting to determine the controlling mechanisms involved in the utilization of amino acids for energy production and protein synthesis in Tetrahymena, and to determine the extent, if any, to which they are "wastefully" utilized. This can be accomplished using radioactive compounds and cells grown in glucose and glucose-free media.

Part III Use of Tetrahymena pyriformis for the Determination of Relative Nutritive Values, Amino Acid Assays and for Amino Acid Supplementation Effects.

In applying Tetrahymena pyriformis W to the evaluation of the Relative Nutritive Value (RNV) of proteins, most workers have used a 4-day cell count as a basis of comparison (Rosen and Fernell, 1956; Stott and Smith, 1966; Teunisson, 1961; Helms and Rolle, 1968). Ideally, the criterion for RNV determination should be total or protein nitrogen synthesized by the organism. Since growth is ultimately the resultant expression of the metabolic activities of a cell and reflects the physiological condition, a study of the growth cannot be complete if it is restricted to simple increments of number. A cell can increase in size and at that time or subsequently would or would not divide depending on its physiological and environmental conditions. Studies on the growth of Tetrahymena have sustantiated that the mean cell size of the population changes with time (figure 10), and nutrient supply. Therefore it seems obvious that an expression of growth involving both cell number and cell size would more accurately estimate the protein synthesized. In addition the elutriation technique proposed by Teunisson (1971) could facilitate the electronic cell counting and sizing.

That the duration of the incubation period may be an important factor in determining the RNVs of some proteins is illustrated in Appendix Table V. Even though
in most cases the maximum growth response was equivalent to that at 96hr; yet as illustrated by the cod and herring FPC samples (Appendix figure I) there could be considerable differences. Also it is conceivable that there could be test proteins, such as protein X (Appendix figure I) which would have attained maximum growth much before 96 hr and would be on the decline or death phase by 96 hr. Therefore it is possible that the use of merely one incubation period could result in an erroneous RNV.

Of the proteins evaluated in the study (Table 7), the RNVs for the faba bean protein concentrate and isolate and the fish protein samples (herring FPC and cod frames) disagreed with the literative values for protein quality. In comparison with the protein indices of faba bean and casein determined with rats, the RNV of faba bean seems to have been overestimated by Tetrahymena pyriformis. Undoubtedly this resulted from a significantly lower sulphur amino acid requirement of Tetrahymena pyriformis compared to the rat and the fact that methionine plus cystine are the limiting amino acids in faba bean. Thus, as has been pointed out elsewhere (Rolle and Eggum, 1970), Tetrahymena pyriformis W may be unsuitable as a test organism for the evaluation of proteins in samples low or limiting in the sulphur amino acids. Since the amino acid composition of cod and herring seem to satisfy the requirements of the organism it was surprising that they were ranked as inferior quality proteins. However, it is

universally recognized that the nutritive values of fish samples vary considerably and that this variation may be attributed either to the initial differences in the raw materials or to the deleterious effects of processing, handling and storage (Ousterhout and Snyder, 1962). It is possible too, considering the repugnant odor, that these fish samples contained rancid fats or even heat stable toxins produced by contaminating bacteria, which repressed the growth of Tetrahymena pyriformis. Boyne et al, 1961 also reported on the inability of Tetrahymena pyriformis to predict the nutritive values of fish protein concentrates by noting low correlations between Tetrahymena evaluations (Tp) and the Gross Protein Values (GPV) of chicks (r= 0.07:GPV/Tp) and NPU of rats (r= 0.10:NPU/Tp) compared to a high correlation between the chick and rat values (r= 0.92:GPV/NPU). The poor quality protein, zein, produced a limited growth response for Tetrahymena pyriformis. This inferior quality of zein, which is also expressed in other feeding trials regardless of the test animal, is thought to be due to the deficiency of the amino acids lysine and tryptophan and also its low solubility.

The Relative Nutritive Values of the other proteins in Table 7 agreed reasonably well with their corresponding published protein values. It is interesting to note the high correlation (r = 0.96) obtained between the <u>Tetrahymena</u> RNVs of this study and the rat Protein Index values (McDonald and Larter, 1974) of the same CIMMYT cereal

samples. Definitely, these results could be enhanced by conducting similar parallel evaluations using <u>Tetrahymena</u> and rats on a larger array of test proteins which have been subjected to similar treatments and storage time. The cost of conducting rat feeding trials on a large number of proteins was considered to be too prohibitive for this particular study.

Generally, the assay of casein, whole egg and faba bean protein concentrate for isoleucine, lysine, threonine and tryptophan (Table 10) yielded values in reasonable agreement with literature values. However, the available lysine and threonine contents of whole egg protein and casein seemed to be under-estimated, considering the high nutritive values of these proteins. Even though an availability of 135% for isoleucine in faba bean concentrated appeared anomalous, it is interesting to note that a microbiologically determined isoleucine content of faba bean of 5.5 mg/100mg protein giving an availability of 137% appears in the literature (FAO, 1970). Unfortunately the significance of this FAO value cannot be fully assessed since a shortcoming of this FAO publication is that the test microorganism was not identified. Of course, availabilities greater: than 100% might be caused by stimulating effects of components other than the amino acid being assayed.

A major contribution of microorganisms to amino acid analysis has been their use for the assay of tryptophan which until recently (Lunver, 1968) could not be determined chemically due to its destruction during acid hydrolysis. The available tryptophan contents of casein and whole egg agreed within 80% of the literature values. The <u>Tetrahymena</u> available tryptophan content of faba bean concentrate, 0.92mg/ 100mg protein, was in close agreement with the literature microbiological value of 0.86mg/100mg protein. This leads

to the speculation that, based on the results of methionine supplementation of faba bean and its higher availabilities of lysine, threonine, isoleucine and possibly tryptophan compared to whole egg and casein, potentially faba bean is a high quality protein.

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The amino acid assays of the CIMMYT cereals (Table 9) also yielded acceptable results. The lower available threonine and sulphur amino acid contents of the inferior quality CIMMYT wheat 4859 and 4860 compared to the CIMMYT triticale samples 4861, 4862 and 4863 were noteworthy. Also a common feature of the CIMMYT cereals in these results is the uniformly high availability of lysine.

Based on a comparison between the chemically determined amino acid contents of the CIMMYT cereals (Appendix Table II) and the amino acid requirement of <u>Tetrahymena pyriformis</u> W (Appendix Table III), lysine would be expected to be the first limiting amino acid followed by threonine. However, based on a comparison between the amino acid content available to the organism (Table 9) and its requirement for that amino acid, threonine became the most limiting amino acid for the two CIMMYT wheat 4859 and 4860 and the CIMMYT triticale 4861. This, in part, could explain the failure of lysine supplementation of the two wheats and 4861 triticale to affect growth. Thus the depression in growth of the organism observed by supplementing CIMMYT wheat 4859 and 4860 with lysine (figure 9 and Table 13) might have been caused by an amino acid imbalance brought

about by supplementation of the second limiting amino acid (Harper, 1964). On the other hand, for the CIMMYT triticale 4861 lysine was just as limiting as threonine or the most limiting amino acid. As expected, lysine supplementation of CIMMYT triticales 4861, 4862 and 4863 produced increased growth responses and thus improvements in the nutritive values.

The reduction in the nutritive values of all five CIMMYT cereals with DL-methionine supplementation seemed to be caused by the toxic effect of methionine which increased with increasing levels of supplementation. It was noted that the severity of the toxic effect increased with decreasing protein quality of cereals. The trend is compatible with the results of Muramatsu et al (1972) who reported that the growth depression of rats fed excessive amounts of a single amino acid was more severe in the low quality protein, wheat gluten, compared to that in the casein and egg albumen diets. It was observed also that the adverse effects of methionine supplementation were reduced by L-lysine HCl supplementation of the CIMMYT triticales 4861, 4862 and 4863. However, in the CIMMYT wheat 4860 supplementation with L-lysine HCl in addition to DL-methionine caused a further depression in the growth response of the organism. The difference in effects of methionine and lysine supplementation was most likely due to the fact that lysine was either most limiting or nearly so in CIMMYT triticales 4861,4862 and 4863. However, it was definitely not the most limiting amino acid in

CIMMYT wheat 4859. Thus according to the definition of amino acid imbalance (Harper, 1964), the toxic effect of an amino acid supplement is expected to be reduced when the quantity of the limiting amino acid is increased. The result of the lysine and methionine supplementation of CIMMYT wheat 4860 could not be explained on this basis.

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The effect of DL-methionine supplementation in improving the protein quality of faba bean concentrate, as assessed by <u>Tetrahymena</u> (Table II and figure 8) confirmed that methionine is the most limiting amino acid in the protein. Assuming almost complete availability of the sulphur amino acid in faba bean, then the highest supplement of 1.0mg DL-methionine/ml would satisfy the organism requirement. Similar results on the improvement of the nutritive value of faba bean concentrate by DLmethionine supplementation have been reported (McDonald and Larter, 1974; Wilson <u>et al</u> 1972) in rat feeding trials.

The fact that tryptophan supplementation (Table 12) failed to improve the RNVs of cod frames and herring FPC suggest that this amino acid is not the most limiting in these samples.

Having applied <u>Tetrahymena</u> <u>pyriformis</u> W to the evaluation of protein quality and the determination of amino acid availabilities, a major question could be posed. Considering the fundamental morphological and physiological differences between ciliates and mammals and the fact that the nutritive value of a protein has

exact significance only in terms of a selected physiological response to a given species, how useful are Tetrahymena evaluations in terms of animal and human nutrition? Obviously there can be no assurance that the RNV of an unknown protein determined by Tetrahymena pyriformis will reflect its biological value for humans. However, it should be pointed out that the various drawbacks of microbiological methods and the far cry from protozoa to mammals are not of great importance if it is recognized that these values are indirect comparative indices instead of absolute measures of protein quality. Thus they should not be applied to human or animal nutrition without further confirmation. Tetrahymena protein quality assays, and indeed other microbiological protein quality assays, have been developed as rapid screening techniques for large numbers of samples. As Rosen (1960) has pointed out, the contribution of microbiological assays of protein quality to progress will be assessed not by their ability to parallel what we know but by the accuracy of their predictions and by the real economics affected in experimentation with higher animals. At present these assays represent a useful link between the rapid physiochemical tagging of protein values that are used in controlling purchasing, processing and plant breeding and the more time-consuming biological evaluations with laboratory or farm animals.

## SUMMARY AND CONCLUSIONS

#### SUMMARY AND CONCLUSIONS

The first part of this study dealt with determining the effects of varying concentrations of different carbohydrates on the growth response of Tetrahymena pyriformis W to different nitrogen sources. The carbohydrates tested were glucose, soluble starch, dextrin and a glucose/starch mixture (1:1) at concentrations from 2.5 mg/ml (0.25%)to 20.0 mg/ml (2%). The nitrogen sources included the intact casein and whole egg proteins, the acid hydrolysate of casein supplemented with L-tryptophan and a crystalline amino acid mixture representing the amino acid requirement of the organism, all supplied at the isonitrogenous concentration of 0.3 mgN/ml medium. Cell counts, mean cell sizes, total population volumes and growth rates were measured, using a Coulter Counter, at 12hr intervals up to 120 hr incubation period. The results indicated that at concentrations equal to or greater than 5.0 mg/ml medium, the polysaccharides soluble starch and dextrin stimulated growth responses equivalent to glucose in the casein medium but higher than glucose in the whole egg protein medium. However, for the protein hydrolysate and the amino acid mixture glucose was superior to the polysaccharides in promoting growth. The growth of the organism was repressed in the early stages by the high glucose concentration of 20.0 mg/ml. It was found that of glucose, dextrin, starch and the glucose/starch mixture at 10.0 mg/ml , the latter was the only carbohydrate

source which stimulated the highest growth response in both the whole egg protein and the amino acid media. This suggested the necessity for the simultaneous presence of glucose and starch when comparing the growth responses of <u>Tetrahymena pyriformis</u> W to intact proteins and to amino acid media, such as in amino acid assays. The carbohydrate concentrations which resulted in optimum growth responses were within the range 5.0 and 20.0 mg/ml for media with 0.3 mgN/ml. The effect of different carbohydrate sources varied with the form (intact vs free amino acid) of the nitrogen source.

Statistical analyses of these results indicated that the carbohydrate level produced significant (P=0.05) effects on the mean cell size, maximum cell count, maximum total volume and growth rate of <u>Tetrahymena pyriformis</u> W. Similar significant effects were observed by varying the protein or nitrogen source in the growth medium. It was found also that the protein/carbohydrate type and protein/ carbohydrate level interactions significantly affected the maximum cell count and the maximum total volume. On the other hand , the effect of varying the type of carbohydrate was significant only when the maximum total volume and the growth rate in the intact protein medium were compared to those in the amino acid medium.

Various hypotheses were discussed for explaining these effects, viz. the deleterious effect of osmotic pressure on the growth of the organism; the effect of

different types of carbohydrates on the metabolism of essential amino acids; the stimulatory effects of particles in the growth medium; and differences in the rates of uptake and availabilities of different energy and nitrogen sources. Of these, the latter hypothesis was suggested as being the most likely to explain the observed effects of various carbohydrate types and concentrations and protein or nitrogen sources.

The second major part of the study dealt with the use of Tetrahymena pyriformis for the determination of Relative Nutritive Values (RNV) and for the assay of amino acid availabilities. The proteins evaluated were casein (RNV = 100), whole egg protein (135), soy protein concentrate (106), faba protein concentrate (59), faba protein isolate (56), herring protein concentrate (41), cod frames (53), zein (3), Casamino Acids (67), amino acid mixture, F (83), CIMMYT wheat 4859 (48), CIMMYT wheat 4860 (53), CIMMYT triticale 4861 (63) CIMMYT triticale 4862 (75), CIMMYT triticale 4863 (74). The <u>Tetrahymena</u> values correlated reasonably well with those reported in the literature except for the faba bean samples which were overestimated. The inability of Tetrahymena pyriformis W to predict the biological nutritive value of faba bean was thought to be related to the organism's low requirement for the sulphur-containing amino acids even though these amino acids were below the organism's requirement in faba bean.

The CIMMYT cereals were assayed for available lysine, threenine and methionine plus cystine. Casein, whole egg protein and faba protein concentrate were assayed for lysine, threenine, isoleucine and tryptophan. The effects of amino acid supplementation showed that the organism does respond positively to increases in the limiting amino acids of the test protein and negatively to amino acid imbalances and toxic levels of certain amino acids. Thus the addition of DL-methionine improved the nutritive values of faba protein concentrate but reduced that of the CIMMYT cereals.

This report suggests consideration of the following recommendations for the Tetrahymena protein quality assays:

- i) <u>Measurement of Growth Response</u> The total cell population volume (= cell count x mean cell volume) would be preferred to cell counts only, as a more accurate estimate of biomass in any expression of protein quality. Since the use of this method employing the Coulter Counter depends on the absence of relatively large undigested food particles from the cell suspension, the elutriation technique of Teunisson (1971) should be further investigated. However, there are cases when cell counts only could suffice, such as in comparing different samples of the same type of protein.
- ii) <u>Incubation period</u> The maximum growth response, as a more meaningful index of the potential

nutritive value of a protein, should be preferred to the growth response merely at one particular incubation period. Thus it is suggested that test protein cultures should be incubated for various times so that the different phases of growth could be identified.

iii) Carbohydrate Source - as part of the general procedure filter-sterilized solution A should be prepared to contain glucose to give a final glucose concentration of 5.0 mg/ml (ie. 0.5%). Pipetting sterile solution A and sterile glucose together, instead of separately, would eliminate an additional opportunity for contamination: solution A (100 x final concentration) + 10 ml 50 ml distilled water, sterilized by filtration + 50 ml , 10% sterile glucose, (20 x final concentration); 1 ml of this mixture should be added (in place of solution A) after autoclaving the medium. When assaying cereals which contain adequate native carbohydrates no additional carbohydrate source is needed other than the glucose contained in solution A. For other nitrogen sources, dextrin or soluble starch should be added to the medium before autoclaving to give a final concentration of 10.0 mg/ml or 1% (ie. 1 ml of 10% polysaccharide suspension prepared at room temperature and added to flasks). A basic assumption of protein quality assays is that amino acids are utilized solely for protein synthesis. However, in the light of the results of the metabolic experiment and the reports of Dewey and Kidder (1973) and Cox <u>et al</u>, (1968) it is apparent that further research is needed to investigate the role of amino acids in energy production in <u>Tetrahymena pyriformis</u> W even in the presence of carbohydrates, and the controlling mechanisms involved in the utilization of these two potential energy sources. This can be accomplished using radioactive substrates.

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# YPPENDIX

Amino Acid Composition of Protein Sources (mg/100 mg protein) Appendix Table I

Zein n/a n/a n/a 2.0 3.0 n/a 3.3 7.3 23.7 n/a n/a I0.9 0.1 n/a n/a n/a 1.7 n/a n/a ł Cod Frames 9.95 5.35 5.93 7.18 4.13 4.46 3.05 1.34 3.39 3.75 6.87 3.65 2.94 6.59 14.01 ll.50 8.23 2.04 7.61 n/a Herring FPC 5.45 4.58 4.63 8.40 4.12 7.29 96.6 4.74 4.92 13.95 6.49 6,80 9.22 2.46 7.48 3.34 3.08 1.50 3.17 n/a Casein hydro. 1.0 1.0 l.0 4.0 5.0 2.0 1.0 3.0 5.0 15.0 5.0 1.5 2.0 2.0 4.0 ·I Т ı ł Soy Conc. G1301 4.60 4.03 5.26 8.13 9.98 l.35 11.28 5.97 5.32 18.52 6.46 3.93 5.57 l.40 1.36 2.76 5.61 4.37 7.67 2.59 Faba iso-late(NVP) 4.65 10.06 0.30 10.59 4.75 13.60 4.36 4.79 3.96 ₫.90 2.39 4.55 9.26 5.41 6.05 8.75 3.94 7.14 0.92 l.47 Faba Con-centrate 4.04. 12.30 18.92 3.65 4.05 9.13 5.02 8.58 0.86 5.67 4.11 0.65 3.56 2.70 9.02 4.7l 0.67 1.32 6.9l 3.51 Casein 3.14 11.28 l.65 7.28 6.16 2.02 8.29 2.98 3.82 4.75 6.88 2.85 9.37 3.22 5.52 9.71 5.34 5.94 11.31 22.50 Whole -Egg 9.62 7.65 4.16 12.74 5.92 6.85 3.36 2.42 5.78 6.29 8.82 4.16 9.89 1.49 3.31 6.98 2.43 6.10 5.12 5.73 Aspartic Acid lotal sulphur Total aromat-Phenylainine soleucine Tryptophan 1ethionine Glutamine acid Glycine Amino Acid listidine Threonine Tyrosine Arginine ic a.a. Proline Alanine Cystine Leucine Jaline Serine ysine a.a

## Appendix Table II

Amino Acid Composition of CIMMYT Wheat and Triticale Samples\* (mg/100 mg protein (defatted samples)

• - ---

Amino Acid	4859 Wheat	4860 Wheat	4861 Triticale	4862 Triticale	4863 Triticale
Lysine	3.1	3.1	3.5	3.4	3.4
Tryptophan	1.7	1.8	1.2	1.1	1.1
Isoleucine	3.8	3.9	3.8	3.9	3.7
Leucine	7.2	7.2	6.8	7.2	6.8
Cystine	2.0	2.2	1.5	1.8	2.0
Methionine	1.8	1.9	1.6	1.9	1.9
Histidine	2.5	2.6	2.5	2.6	2.4
Arginine	5.8	5.6	6.3	6.1	6.4
Tyrosine	3.6	3.7	3.2	3.4	3.3
Phenylalańińe	4.8	4.9	4.8	5.0	4.9
Threonine	3.1	3.2	3.0	3.2	3.0
Valine	4.7	4.8	4.6	4.9	4.6
Aspartic Acid	5.5	5.6	6.3	6.5	6.3
Glutamic Acid	36.0	37.0	31.9	33.4	32.4
Proline	9.9	10.0	9.8	9.9	10.0
Alanine	3.9	3.9	4.0	4.2	4.0
Serine	5.0	4.9	4.4	4.6	4.5
Glycine	4.6	4.6	4.2	4.5	4.3

\* Data supplied by private communication from Dr. E. Mertz, Purdue University.

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### Appendix Table III

\* Solution F - Crystalline Amino Acid Mixture based on the

requirements of <u>Tetrahymena</u> pyriformis W

Amino Acid	(10 x final strength) mg/100 ml or mg/300mgN	mg/16mg N
L- Lysine HCl	180.0	9.60 (lys 7.70)
L - Histidine HCl	62.0	3.31 (his 2.69)
L - Arginine HCl	136.0	7.25 (arg 6.01)
L - Thre <b>o</b> nine	106.0	5.65
Er - Valine	141.0	7.52
DL- Methionine	34.0	1.8
L - Cystine		_
L - Isoleucine	117.0	6.24
L - Leucine	164.0	8.75
L - Phenylalanine	118.0	6.29
L - Tyrosine		-
L - Tryptophan	31.0	1.65
L - Aspartic Acid	252.0	13.44
DL- Serine	256.0	13.65
L - Proline	109.0	5.81
L - Glutanic Acid	335.0	17.87
LGlycine	87.0	4.64
DL - Alanine	155.0	8.27

\* Private Communication Dr. E. B. Smith, Foods and Nutrition Department, University of Manitoba.

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Stock Solution A (100 x final	strength)	(mg/200ml)
Calcium pantothenate	12.5	
Nicotinamide	12.5	
Pyridioxine hydrochloride	125.0	
Pyridoxal hydrochloride	12.5	
Pyridoxanine hydrochloride	12.5	
Riboflavin	12.5	
Folic acid	1.25	
Thiamine hydrochloride	12.5	
Inositol	12.5	
Choline Chloride	125.0	
p- Amino benzoic acid	12.5	
Biotin	1.25	
DL-«-Lipoic Acid	0.4	
Stock Solution B (100 x final	strength)	(g/200ml)
MgSO <sub>4</sub> . 7H <sub>2</sub> 0	2.8	
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> .6H <sub>20</sub>	1.25	
MnCl <sub>2</sub> .4H <sub>2</sub> 0	0.025	
ZnCl <sub>2</sub>	0.0025	
Stock Solution C (100 x final	strength)	

CaCl<sub>2</sub>.2H<sub>2</sub>O mg/200ml)

CuCl<sub>2</sub>.2H<sub>2</sub>O

FeCl<sub>3</sub>.6H<sub>2</sub>O

Stock Solution D (100 x final strength) (g/200 ml)

<sup>KH</sup> 2 <sup>PO</sup> 4	3.5
K2 <sup>HPO</sup> 4	3.5

Stock Solution E (5 x final strength)

(The appropriate weights of the components listed below are dissolved in approximately low1 distilled water, lml stock solutions B, C, D added and the whole made up to 20 ml

600

60

	mg/20ml Solution E
Guanylic acid (Na salt)	15
Adenosine-2'(3')-phosphoric	10
Acid monohydrate	10
Cytidylic acid	12.5
Uracil	5

Appendix Table V The effect of using cell count or total population volume at different incubation periods on the Relative Nutritive Values of Protein Sources using <u>Tetrahymena</u> pyriformis W

PROTEINS	RNV usi at	RNV calculated using cell counts at			RNV calculated using total pop- ulation volumes at			
	72h	96h	Max	72h	96h	Max		
Casein	100	100	1.00	100	100	100		
Egg, whole	186	165	139	204	115	135		
Soy, conc.	107	111	96	90	101	106		
Faba, conc.	73	554	48	113	60	59		
Faba, isol.(nvp)	82	70	62	97	61	56		
Herring, FPC	26	33	46	15	28	41		
Cod, frames	45	65	63	44	51	53		
Zein	4	6	4	3	3	3		

#### Appendix Table VI

Analysis of Variance. 3 Factor Experiment Randomized Block Design. Part la. Mean Cell Volumes ( $\mu^3 \times 10^3$ ) of <u>Tetrahymena pyriformis W</u> grown in whole egg protein, casein and casein hdyrolysate media with varying levels (0-20.0mg/ml of glucose or starch)

		·						
	Carb. Type (T)	Gluc	ose		Starc	Starch		
	Prot- ein .(P)	Casein	Egg	Casein Hydrol.	Casein	Egg	Casein Hydrol	Total
	1 <u></u> 0	18.3	25.5	22.9	18.3	25,5	22.9	133.4
	2.5 mg/1	23.0	30.3	28.5	25.9	24.6	30.6	162:9
	(I) 5.0	29.1	28.1	31.7	32.9	31.5	31.7	185.0
	pohj 0.01 s 0.0	29.4	31.0	31.7	29.0	34.2	33.0	· 188.3
	Carl Carl Carl	33.0	35.2	32.3	29.5	30.0	31.6	191.6
	Total	132.8	150.1	147.1	135.6	145.8	149.8	861.2
	Correction Ter	rm	24722.	2			ļ	
	Total Levels:		861.	2				
	Total Glucose:	:	430.	0				
	Total Starch:	,	431.	2				
	Total Casein:		268.	4				
	Total Egg:		295.	9				
	Total Casein h	nydrolysate	296.	9				
	Source of Vari	Lation	df	SS	MS	f	<sup>F</sup> .05	
	Protein source	e, P	2	52.3	26.2	8.24*	4.46	
	Carbohydrate t	cype, T	l	0.03	0.03	21	5.32	
	Carbohydrate ]	level, L	4	398.6	99.7	31.4*	3.84	
•	Interaction, I	тç	2	3.3	1.65	≤1	4.46	
	" E	PL ·	8	47.0	5.88	1.85	3.44	
	" J	ſL	4	26.2	6.55	2.06	3.84	
†	Error		8	25.4	3.18			
	Total		29	552.8				

+ 3 factor interaction term PTL used to estimate error

## Appendix Table VII

Analysis of Variance. 3 Factor Experiment, randomized block design. Part la. Maximum cell counts (x  $10^4/ml$ ) of <u>Tetrahymena pyriformis</u> <u>W</u> grown in whole egg protein, casein and casein hydrolysate media with varying levels (0-20.0 mg/ml) of glucose or starch.

-	Carb. Type (T)	( + )	Glu	cose .		Star	• •		
-	Prot- ein		Casein	Egg	Casein Hydrol	Casein	Egg	Casein Hydrol	Total
	н	0	33.0	47.8	22.2	33.0	47.8	22.2	206.0
	re ng∕m	2.5	68.4	99.4	47.6	68.6	98.6	33.8	416.4
	drat (L)n	5.0	74.7	94.7	48.6	72.3	109.6	35.9	435.8
	ohy 1s	10.0	76.3	84.4	46.5	78.3	108.5	38.5	.432.5
	Carb Leve	20.0	62.9	68.8	48.4	59.7	90.4	36.0	366.2
	Tota	11	315.3	395.1	213.3	311.9	454.9	166.4	1856.9
Correction Term: 11,4935.9									
ŗ	Total	Levels:		185	56.9				
	F3	Glucose:		<b>9</b> 2	23.7				
" Starch:				93	33.2				
	17	Casein:		62	27.2				
	17	Egg:		85	50.0				
	11	Casein H	lydrolysat	e: 37	79.7				
	Source	e of Vari	ation	df	SS	MS	f	<sup>F</sup> 0.05	
	Protei	in source	e, P.	2	11035.3	5517.7	196.4*	4.46	
(	Carboł	nydrate t	уре Т	1	3.02	3.02	<1	5.32	
	H .	I	evel, L	4	6214.9	1553.7	55.3*	3.84	
	Intera	action PI	1 -	2	609.7	304.9	10.9*	4.46	
	11	PI	2	8	1008.4	126.1	4.49*	3.44	
	17	TI	4	4	92.2	23.1	<1	3.84	
	+Erroi	5		8	224.5	28.1			
I	Total			29	19188.0				
+ 3 factor interactio			eraction	term PTT.	used to	estimate	error		

\* significant at P = 0.05

Appendix Table VIII

Analysis of Variance 3 Factor Experiment, Randomized Block Design. Part Ia. Maximum total volume  $(\mu^3 x l 0^9)$  of <u>Tetrahymena pyriformis</u> W grown in whole egg protein, casein and casein hydrolysate media with varying levels (0-20.0mg/ml of glucose or starch.)

		·						1
Carb. Type	~	Gluc	Glucose			Starch		
Prot- ein	j	Casein	Egg	Casein Hydrol.	Casein	Egg	Casein Hydrol	Total
	0	4.1	7.2	3.1	4.1	7.2	3.1	28.8
[m/	2.5	9.7	14.6	6.3	9.7	15.4	6.5	62.2
cate L)mg	5.0	11.0	15.0	7.6	9.9	16.1	7.7	67.3
hydi s (J	10.0	14.4	15.7	9.6	13.9	19.6	7.7	.80.9
Carbo Level	20.0	14.2	19.8	8.7	12.3	19.0	6.6 .	80.6
Tota	al	53.4	72.3	35.3	49.9	77.3	31.6	319.8
Correc	ction Ter	rm :	3409	.1				
Total	Levels:		319	. 8				
U	Glucose	•	161.	.0				
11	Starch:		158	.8.				
н	Casein:		103	.3				
11	Edd:		149	<b>.</b> 6				
;1	Casein 1	hydrolysat	e: 66	.9				
Sourc	e of Var	iation	df	SS	MS	f	<sup>F</sup> .05	
Prote	in sourc	e, P	2	343.6	171.8	229.1*	4.46	
Carbo	hydrate	type, T	1	0.13	0.13	<1	5.32	
11		level, L	4	302.4	75.6	100.8*	3.84	

4.97

34.6

4.27

6.03

696.0

3.32

5.77\*

1.43

2.49

4.33

1.07

0.75

4.46

3.44

3.84

+ 3 factor interaction PTL used to estimate error

2

8

4

8

29

\* significant at P = 0.05

PL

TL

Interaction, PT

п

†<sub>Error</sub>

Total

#### Appendix Table IX

Analysis of Variance. 3 Factor Experiment Randomized Block Design. Part Ia Growth Rate  $(hr^{-1})$  of <u>Tetrahymena pyriformis</u> W grown in whole egg protein, casein and casein hydrolysate media with varying levels (0-20.0 mg/ml) of glucose or starch.

·····						······		
Carb. Bype (T)		Gluco	ose .		Starch			•
Pro- tein (P)		Casein	Egg	Casein Hydro.	Casein	Egg	Casein Hydrol.	Total
(	0	0.13	0.14	0.13	0.13	0.14	0.13	0.80
re g/ml	2.5	0.14	0.18	0.14	0.16	0.17	0.16	0.95
drat L)mg	5.0	0.16	0.21	0.15	0.16	0.23	0.17	1.08
v v v v v v v v	0.0	0.16	0.20	0.14	0.15	0.18	0.19	1.02
Carb Level	0.0	0.13	0.17	0.12	0.15	0.18	0.18	0.93
Fotal		0.72	0.90	0.68	0.75	0.90	0.83	4.78
Correct:	ion Te	erm:	0.762					
Total Le	evels:		4.78					
" G	lucose	2:	2.30					
" St	tarch:	· _	2.48					
" Ca	asein:	:	1.47					
" Eq	33:		1.80					
" Ca	asein	hydrolysate	e: 1.51					
Source	of Vai	ciance	df	SS	MS	f	<sup>F</sup> .05	
Protein	, P		2	0.006	0.003	5.0*	4.46	
Carbohy	drate	Туре, Т	1	0.001	0.001	1.67	5.32	
n		Level, L	4	0.006	0.006	3.33	3.84	
Interac	tion I	тç	2	0.001	0.0005	<1	4.46	
11		5F	8	0.004	0.0005	<1	3.44	
u	r	- ГL	4	0.001	0.0003	< <u>1</u>	3.84	
Error			8	0.005	0.006			
Total			29	0.024				

+ 3 factor interaction term \* significant effect

PTL used to estimate error
## Appendix Table X

Analysis of Variance. 2 Factor Experiment, Randomized Block Design. Part I b. Mean Cell Volume of <u>Tetrahymena pyriformis</u> W grown in whole egg protein or solution F media with different carbohydrate sources (values are totals of 3 replicates of mean cell volume in  $\mu^3 x 10^3$ )

	-					
			Carbohydra	ate Type (T	)	
		Glucose	Starch	Dextrin	Glucose/ Starch	Total
л (Р)	Egg	107.1	112.2	99.6	108.6	427.5
Protei	F Eran	93.3	108.9	105.3	102.6	410.1
	Total	200.4	221.1	204.9	211.2	837.6

Correction Term: 29232.2

Source of Variation	df	SS	MS	f	F .05
Protein:P	1	12.7	12.7	2.31	4.49
Carbohydrate type:T	3	40.3	13.4	2.44	3.24
Interaction: PT	3	32.3]	10.8	1.90	3.24
Error	16	88.6	5.5		

## Appendix Table XI

Analysis of Variance. 2 Factor Experiment Randomized Block Design. Part 1b. Max Cell Count of <u>Tetrahymena pyriformis</u> W grown in either whole egg protein or solution F media with different carbohydrate Source at a constant level 10.0 mg/ml (Values are totals of 3 replicable counts in cells x 104/ml)

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			Carbohydr	ate Type (T	)	
		Glucose	Starch	Dextrin	Glucose/ Starch	Total
n (P)	Egg	253.2	325.5	310.2	328.8	1217.7
Protei	F	174.3	91.2	168	184.8	618.3
	Total	427.5	416.7	478.2	513.6	1836.0

Correction Term: 140454

Source of Variation	df	SS	MS	f	<sup>F</sup> .05
Prot <b>ein,</b> P	1	14970.0	14970.0	2.04	4.49
Туре, Т	3	1021.8	340.6	<1	3.24
Interaction, PT	3	2043.2	681.1	<1	3.24
Error	16	117573.0	7348.3		

Appendix Table XII

Analysis of Variance. 2 Factor Experiment Randomized Block Design. Part Ib. <u>Max. total volume of Tetrahymena pyriformis</u> W grown in whole egg protein or solution F media with different carbohydrate sources. (Values are totals of 3 replicates max. total volumes in  $u^3x10^9/ml$ .

•						
	x		Carbohydı	cate Type (	r)	
		Glucose	Starch	Dextrin	Glucose/ Starch	Total
(P)	Egg	47.1	58.8	47.7	58.5	212.1
Prote:	F	33.6	25.8	32.4	35.4	127.2
	Total	80.7	84.6	80.1	93.9	339.3

Correction Term: 4796.9

Source of Variation	df	SS	MS	f	<sup>F</sup> .05
Protein, P	1	300.3	300.3	195.0*	4.49
Туре, Т	3	20.3	6.8	4.42*	3.24
Interaction, PT	3	39.5	13.2	8.57*	3.24
Error	16	24.6	1.54		

Appendix Table XIII

Analysis of Variance. 2 Factor Experiment Radomized Block Design. Part Ib. Growth Rate (hr<sup>-1</sup>) of <u>Tetrahymena pyriformis</u> W grown in either whole egg protein or solution F media with different carbohydrate sources at a constant level 10.0mg/ml.

		۱ <u></u>					
		(	Carbohydrat	е Туре (Т)			
		Glucose	Starch	Dextrin	Glucose/ Starch	Total	
1 (P)	Egg	0.60	0.57	0.69	0.60	2.46	
Protei	F	0.63	0.57	0.60	0154	2.34	
	Total	1.23	1.14	1.29	1.14	4.80	
Corr	ection T	erm:	0.003				
Sour	ce of Va	riation	df	SS	MS	f	F

Protein, P	l	0.001	0.001	3.33	4.49
Carbohydrate Type, T	3	0.002	0.001	3.33*	3.24
Interaction, PT	3	0.0	0.0	<1	3.24
Error	16	0.004	0.0003		

\* significant at P = 0.05

Appendix Table XIV

Summary Table of Analysis of Variance of Data from Part Ia and Part Ib. Figures are the calculated f values.

and the \* indicates significance at P = 0.05

				· • • • • • • • • • • • • • • • • • • •			-
	Part Ia Protein hydroly Carbohy Carbohy	s: Whole egg, c sate 0.3mgH/ml drate Types: gl drate levels: 0, 20	asein, casein ucose, starch 2.5,5.0,10.0, .0mg/ml		Part 'Ib. Proteins: bohydrate starch, g	Whole E Types: c rlucose al	gg, Solution F, Car- glucose, dextrin, nd starch 10.0mg/ml.
	Protein P	Carbohydrate Type T	Carbohydrate Level L	Interactions PT PL TL	ρ	. E4	Interactions
Mean Cell Volume	8.24*	۲ ٦ ۲	31.4*	<1 1.85 2.6	2.31	2.44	1.96
Max Cell Count	196.4*	г. >	55.3*	10.9* 4.49* <1	2.04	<1	۲۱
Max Total Volume	229.1*	< 1	100.8*	3.32 5.77* 1.43	195.0*	4.42*	8.57*
Growth Rate	5°0*	1.67	3.33	<1 <1 <1	3.33*	3.33*	۲۶

Appendix Figure I:

## Growth of Tetrahymena pyriformis W in

different protein media.



Appendix Figure II: Protein/carbohydrate Level (PL) interaction effect on the maximum total population volumes of <u>Tetrahymena pyriformis W</u>, based on statisticaly analysis of variance of Part la. results.



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Appendix Figure III: Protein/carbohydrate Level (PL) interaction effect on the maximum cell count of <u>Tetrahymena pyriformis W</u> based on statistic analysis of variance of Part 1 a. results



Appendix Figure IV.

Protein/carbohydrate type (PT) interaction effect on the maximum cell count of <u>Tetrahymena pyriformis W</u> based on statistica analysis of variance of Part 1 a results.

