THE UNIVERSITY OF MANITOBA

STUDIES ON TETRAHYMENA PYRIFORMIS W. EFFECTS OF CARBOHYDRATE ON GROWTH AND USE OF THE ORGANISM IN PROTEIN QUALITY EVALUATION.

by

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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

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 Tetrahymena pyriformis 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 ($\log_{10}$ 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 Tetrahymena pyriformis 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, dextrin and 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 Index values from rat feeding experiments. The RNVs of 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

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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 Tetrahymena pyriformis 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: -

1) To determine the effect of different types and concentrations of carbohydrate on the growth responses of Tetrahymena pyriformis W in media containing different types of nitrogen sources.
2) 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 *Steptococcus faecalis*, *Streptococcus zymogenes* and *Leuconostoc mesenteroides* and the protozoan, *Tetrahymena*, 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 Tetrahymena 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 Tetrahymena 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, Tetrahymena pyriformis 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 Tetrahymena 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 Tetrahymena pyriformis 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

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_3 - 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:

$$RNV = \frac{\text{Log}_{10}(\text{cell count for test protein}) - \text{Log}_{10}(\text{cell count for inoculum})}{\text{Log}_{10}(\text{cell count for standard}) - \text{Log}_{10}(\text{cell count for inoculum})} \times 100$$

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 cell-count.

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