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Microbiological evaluation of protein quality with *Tetrahymena pyriformis* W

1. Characteristics of growth of the organism and determination of relative nutritive values of intact proteins

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In assays of protein quality, factors that determine the nutritive value of a protein are not the same for all animal species. The term, nutritive value of a protein, has exact significance only in terms of a selected physiological response in a given species. In some circumstances, however, animal feeding trials are too time-consuming and expensive, and indirect or empirical methods have to be employed.

Of the many factors affecting protein quality, the essential amino-acid composition is considered to be the most fundamental. According to the 'limiting amino-acid concept' the nutritive value of a protein is determined by the availability of the most deficient essential amino-acid as judged by the requirements of the animal. Block & Mitchell (1946–7) have rated proteins on a 'chemical score' basis whereby the amino-acid composition of a protein is compared with the composition of whole-egg protein, claimed to be completely utilized in the nutrition of the growing rat. The chemical score is the percentage deficit in the limiting essential amino-acid subtracted from 100. Despite a high degree of correlation with 'protein efficiency ratios' and 'biological values' for the growing rat, Block & Mitchell point out that perfect correlation cannot be expected.

It is known that the order of release of amino-acids and the extent of hydrolysis play an important part in the protein nutrition of an animal and, in relating the results of chemical and microbiological assays of amino-acids to the requirements of a particular species, the assumption is made that the total amino-acid complement is available for

assimilation by the animal. In order to avoid the destructive effects of chemical hydrolysis on certain amino-acids, enzymic digestion has been employed as a prelude to microbiological assay of individual amino-acids. To simulate more closely the fate of proteins in vivo, Halevy & Grossowicz (1953) used in vitro digestion with pancreatin and assayed the released amino-acids as a group, using a strain of *Streptococcus faecalis* requiring the ten amino-acids considered essential for the growing rat. The degree of hydrolysis obtained by this procedure was low and the objection may be raised that hydrolysis and assimilation are not concurrent.

The comprehensive studies of Kidder & Dewey (1951) on the nutritional requirements of the protozoon, *Tetrahymena pyriformis*, have revealed the potentialities of the organism as an experimental animal. It is a ciliated protozoon, which can be grown in pure culture on a chemically defined medium, and its requirements are similar in many respects to those of higher animals. The most important features of the species (strain W) as an organism for the microbiological assay of protein quality are its requirement for the same amino-acids as the growing rat and its ability to digest protein.

Rockland & Dunn (1949) used *T. pyriformis* H to assay protein quality, taking acid production over 41 days as an index. Anderson & Williams (1951) proposed a colorimetric method for the determination of growth of *T. pyriformis* W on amino-acid media and also reported response curves for growth on four proteins. More recently, Pilcher & Williams (1954) and the present authors (Fernell & Rosen, 1954; Rosen & Fernell, 1954) have described microbiological assays for the determination of protein quality. Whereas Pilcher & Williams used growth in relation to food nitrogen as a basis for the comparison of proteins, we proposed the use of growth in relation to ammonia N production as an index of the efficiency of protein utilization.

This paper gives an account of the growth and metabolism of *T. pyriformis* on proteins and the determination of relative nutritive values of partially purified proteins. The next paper (Rosen & Fernell, 1956) deals with the application of the assay to foodstuffs. Together they describe the development of a microbiological method for assessing protein quality.

EXPERIMENTAL

The test organism. The organism has been variously described as, e.g., *Tetrahymena pyriformis*, *T. geleii*, *Glaucoma pyriformis*. The nomenclature and systematic status of strains of the *Colpidium-Glaucoma-Leucophrys-Tetrahymena* group of holotrichs have been reviewed by Corliss (1953).

T. pyriformis W was obtained from the Culture Collection of Algae and Protozoa, Botany School, Downing Street, Cambridge.

Maintenance. Stock cultures of the organism were maintained at 25° in 1 oz. screw-capped bottles containing 10 ml. of nutrient broth of the following composition (g/100 ml.): proteose peptone (Difco) 2·0, Yeastrel (Brewer's Food Supply Co. Ltd, Edinburgh) 0·1, glucose 0·5, sodium chloride 0·1, adjusted to pH 7·1 and sterilized by autoclaving (15 lb./sq. in. for 15 min). Stock cultures were transferred at weekly intervals. For the inoculation of experimental media, actively growing 3-day broth

cultures were used. Weekly tests were made for bacterial contamination by 'plating' on nutrient agar.

Basal medium (*Table* 1). The medium was derived from the D medium of Kidder & Dewey (1951) by the omission of amino-acids (replaced by test proteins) and sodium acetate. DL-α-lipoic acid was substituted for protogen. Tween (Honeywill & Stein Ltd, London) was omitted since preliminary trials on proteins gave no evidence of growth stimulation, and Kidder (1953) has since reported that the increased turbidity of cultures on media containing Tween is due to inclusions of fatty droplets in the organisms. Although inositol, choline and *p*-aminobenzoic acid are not absolutely required (Kidder & Dewey, 1951), they were retained in the medium to cover possible sparing effects on the growth of high populations on proteins.

Table 1. *Composition of basal medium*

	Component	Concentration in assay medium (μg/ml.)		Component	Concentration in assay medium (μg/ml.)
(A)	Pantothenic acid	0·625	(B)	$MgSO_4.7H_2O$	140·0
	Nicotinamide	0·625		$Fe(NH_4)_2(SO_4)_2.6H_2O$	62·5
	Pyridoxin hydrochloride	6·25		$MnCl_2.4H_2O$	1·25
	Pyridoxal hydrochloride	0·625		$ZnCl_2$	0·125
	Pyridoxamine hydrochloride	0·625	(C)	$CaCl_2.2H_2O$	30·0
	Riboflavin	0·625		$CuCl_2.2H_2O$	3·0
	Folic acid	0·0625		$FeCl_3.6H_2O$	0·75
	Thiamine hydrochloride	6·25	(D)	K_2HPO_4	175·0
	Inositol	0·625		KH_2PO_4	175·0
	Choline chloride	6·25	(E)	Guanylic acid	150·0
	p-aminobenzoic acid	0·625		Adenylic acid	100·0
	Biotin	0·03		Cytidylic acid	125·0
	Lipoic acid	0·010		Uracil	50·0
			(F)	Glucose	40,000

For convenience sterilized stock solutions (at 100 times final medium) were kept of these groups of components, with the exception of group E which was weighed out as required. To avoid precipitation it is advisable to dilute the concentrated solutions of the mineral salts before mixing.

Protein sources. 'Vitamin-free' casein for these studies was prepared from a commercial sample of New Zealand casein. It was twice reprecipitated at its iso-electric point with 0·05 N-HCl, washed exhaustively with 90% ethanol and finally with absolute ethanol and ether. The solvent was removed at 37°. Other proteins were prepared from commercially available materials.

Preparation of proteins. To avoid as far as possible the influence of non-protein materials, partially purified proteins were used, isolated by the following procedure. Finely ground ether-washed materials (one part) were extracted for 4 h with twenty parts of 0·1 N-sodium-hydroxide solution at room temperature. The clarified extract was adjusted to pH 5·0 with N-HCl and precipitation of the protein completed by addition of five volumes of acetone. The precipitate was then washed once with acetone and twice with diethyl ether, and allowed to dry at 37°. When solvent-free the protein was ground to pass a 72-mesh B.S. sieve. Proteins that did not dissolve readily at pH 7·1 were dispersed into a fine paste and dissolved when possible at pH 8–11. If

the protein was partly insoluble or partially reprecipitated on neutralizing the alkaline solution to pH 7·1, the mixture was ground in a mortar and used as a fine suspension. Dispersions were allowed to stand overnight. After dilution with water to the desired N concentration, the pH was finally adjusted to 7·1. It should be noted that gelatin, commercial egg albumen, fresh egg white and 'vitamin-free' casein were not subjected to this partial purification.

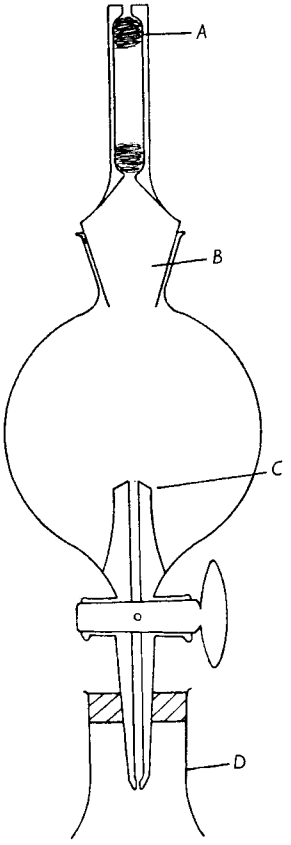

Fig. 1. Inoculation device. *A*, glass tube, packed with non-absorbent cotton wool and joined to a ground-glass stopper *B*. The raised outlet *C* of the separating funnel is designed to ensure that only motile organisms are transferred. *D* is a glass cone, which acts as a shield and locates the neck of the bottle below the delivery tip.

Assay procedure. A total volume of 10 ml. in a 2 oz. 'medical-flat' screw-capped bottle was used, made up of the following, adjusted to pH 7·1:

(1) 4 ml. of medium 2·5 times the final strength, excluding vitamins and glucose.

(2) 4 ml. of protein solution or suspension 2·5 times the final strength.

(3) 1 ml. portions of vitamin mixture and glucose ten times the final strength.

The bottles containing basal medium and protein were autoclaved at 15 lb./sq.in. for 15 min; when they were cool, 1 ml. portions of glucose and vitamin solutions, separately sterilized at 15 lb./sq. in. for 15 min., were added aseptically by means of sterile 1 ml. blow-out type pipettes.

The vitamins and glucose solutions were autoclaved separately to avoid glucose–protein interaction and the possible binding of vitamins to protein.

Each bottle received an inoculum of two drops (approx. 0·06 ml.) of a 3-day broth culture from a device shown in Fig. 1. This is essentially a modified separating funnel, which can be sterilized and aseptically filled with the culture of *T. pyriformis*.

After inoculation, the bottles (with loose screw-caps) were placed in special racks at a slope of 15° to the horizontal, ensuring a high surface:volume relationship of the culture medium. After 4 days' incubation at 25°, the bottles were removed for analysis. Cultures grown in triplicate were combined for analysis.

Sterilization of proteins by filtration. For assays of unheated proteins, centrifuged solutions were sterilized by filtration through a bacterial grade Berkefeld candle under moderately reduced pressure.

Estimation of total nitrogen (Kjeldhal). The digestion procedure of Chibnall, Rees & Williams (1943) was adopted. The distillation procedure was as outlined in the determination of ammonia but with 40% NaOH.

Count. Samples for analysis were withdrawn from the culture after shaking for 2 min in a Microid Flask Shaker (speed 5). One ml. of the culture was removed and

transferred to a $\frac{1}{4}$ oz. screw-capped bottle containing 1 ml. of preserving fluid (a 6% solution of formaldehyde in phosphate buffer of five times the concentration of the D component of the assay medium). The number of organisms was determined microscopically in a Neubauer-ruled double-cell haemocytometer. Organisms in the 1 sq.mm network were counted and the mean of eight determinations multiplied by dilution $\times 10^4$ gave the population per ml. of the original culture. Before counting, the four networks of each cell were inspected to confirm that an even filling had been obtained. It is desirable that two operators should make separate determinations on each sample.

Determination of ammonia. To 6 ml. of the culture were added 4 ml. of 12·5% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifuging and 3 ml. portions of the supernatant liquid were usually taken for analysis by the steam distillation procedure of Markham (1942), substituting saturated potassium carbonate for the concentrated sodium hydroxide. The ammonia was distilled into 10 ml. of 1% (w/v) boric acid. 0·01 N-HCl was used for titration, with a methylene blue–methyl red indicator (Yuen & Pollard, 1953).

Separation of ammonia and glutamine. The procedure was essentially the preferential retention of ammonia by an ion-exchange resin. Amberlite IRC-50 (British Drug Houses Ltd) was used in the Na form in columns of 20 × 1 cm internal diameter containing 10 g wet weight of resin. A 5 ml. portion of the culture fluid, after deproteinization, was washed through the column by three separate 5 ml. portions of distilled water. The fraction passing through the column contained any glutamine present. The ammonia retained on the column was removed by elution with 10 ml. N-HCl and the column washed with small portions of distilled water.

Estimation of protein hydrolysis. The 'formol N' titration was adopted for the investigation of proteolysis.

Portions of from 2 to 5 ml. of the culture fluid were diluted with 30 ml. of distilled water and adjusted to pH 7·0. Neutralized formaldehyde (40% AR) (5 ml.) was added and the mixture titrated to pH 9 with standard alkali (0·02 N). The percentage hydrolysis of the protein was then computed as follows:

$$\text{Hydrolysis (\%)} = \frac{\text{mg 'formol' N/ml.} + \text{mg organism N/ml.}^*}{\text{mg total N/ml.}} \times 100.$$

This estimate is not claimed as a precise measure of proteolysis, but as a simply determined index for comparative purposes.

pH measurement. All measurements were made on a direct-reading pH meter with a glass electrode (Electronic Instruments Ltd, Richmond, Surrey).

RESULTS

Selection of assay conditions

Incubation time. When cultures of *T. pyriformis* were examined over a period of 8 days it was found that on most proteins maximum motile populations were reached on the 5th day. After the 5th day increasing numbers of non-motile or dead organisms

* 10^6 organisms contain 0·22 mg N.

were observed. To ensure that comparisons were made on motile cultures of near-maximal population, the incubation period was limited to 4 days.

Influence of aeration on growth. In preliminary experiments on broth cultures of *T. pyriformis* in tight-capped 2 oz. bottles containing 10 ml. medium, it was found that after the 3rd day an increasing number of lethargic or dead organisms was observed. That their presence was due to a lack of oxygen is supported by the results quoted in Table 2 for cultures at 4 days.

Table 2. *Effect of aeration on the growth of* Tetrahymena pyriformis *on nutrient broth for 4 days*

Arrangement	Condition of organisms	Count (10^6/ml.)	pH
Bottles vertical, tight caps	Some dead and shrivelled	0·22	6·4
Bottles vertical, loose caps	Well formed and alive	0·42	7·5
Bottles sloped, tight caps	Very few alive	0·40	6·1
Bottles sloped, loose caps	Well formed and alive	0·65	7·9

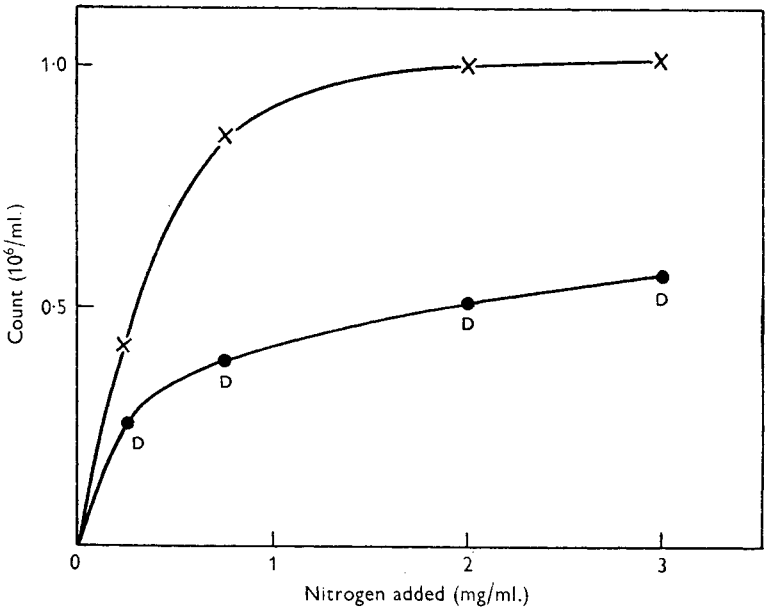

Fig. 2. Effect of oxygen limitation on the growth of *T. pyriformis* on 'vitamin-free' casein for 4 days. ×—×, bottles with loose caps; ●—● bottles with tight caps; D, dead organisms present.

Loose caps and increasing the surface to volume relationship of the culture led to a marked increase in growth. A further illustration is given in Fig. 2 which shows the difference in growth response of *T. pyriformis* in sloped cultures with loose and tight caps at various levels of casein.

Measurement of organism response. Ideally the growth-promoting abilities of various proteins for *T. pyriformis* should be measured in terms of tissue-protein synthesis. The direct approach would be the separation of the organisms and a determination of the N content of the populations attained. Since it was ultimately planned to work on foods containing whole protein, and since even in the work on isolates there was

always a proportion of insoluble food residues present after growth, the direct approach of separating organisms and determining N or protein content was not feasible. Differential centrifuging in sucrose solutions and electromigration techniques were tried without success.

If it is assumed that the composition and the average dry weight of the organism does not vary with the protein used for growth, then the amount of tissue protein synthesized will be proportional to the number of organisms. The usual conventional turbidity procedures cannot give accurate measurements of population density when, as in our investigation, insoluble and coloured materials occur in the medium.

Organism response has been measured by acid production (Rockland & Dunn, 1949) and the enzymic reduction of 2:3:5-triphenyltetrazolium chloride (T.P.T.Z.) (Anderson & Williams, 1951; Pilcher & Willlams, 1954). Acid production is not directly related to the amount of protein synthesis and depends to a large extent on the degree of anaerobiosis. A titration procedure is further complicated by the excretion of ammonia, the end-product of N metabolism. The production of ammonia is often sufficient to raise the pH of the medium above 7·0 and results of a titration as proposed by Rockland & Dunn (1949) would be meaningless. In fact negative acid productions were reported by these authors.

When various proteins were added to portions of a 4-day broth culture immediately before incubation for 4 h at 25° in the presence of T.P.T.Z., the colours produced varied according to the nature of the protein added (Table 3).

Table 3. *Effect of proteins on the enzymic reduction of* 2:3:5-*triphenyltetrazolium chloride by* Tetrahymena pyriformis

Solutions added to broth culture	Colour intensities (EEL colorimeter readings, 621 filter) with quantities of N added of: 0·2 mg/ml.	0·6 mg/ml.	1·0 mg/ml.
Soya protein	51	52	54
Egg albumen	48	44	48
Groundnut protein	35	32	36
Casein	30	26	26

Colour intensity of broth culture + water = 43.

It is of interest that Jámbor (1955), in a recent chemical study on the mechanism of the reduction of tetrazolium salts, states that the rate of reduction was increased by certain colloids, including gelatin.

It was therefore decided to assess the growth response by counting the organisms microscopically.

Determination of relative nutritive values. When *T. pyriformis* was grown on graded levels of protein, the growth obtained did not necessarily accord with the values expected from consideration of accepted nutritive ratings.

Fig. 3 shows a striking example of this behaviour. From 0 to 0·5 mg N/ml. the responses were in the order expected—casein > soya-bean > groundnut—but at the 2–3 mg N/ml. level soya-bean and groundnut supported a much greater population than casein. Anderson & Williams (1951) recorded a similar cross-over of response curves for *T. pyriformis* grown on casein and soya-bean meal.

The most plausible explanation for this behaviour is that differing rates and extents of hydrolysis affect the growth response. Table 4 illustrates the relationship between count and percentage hydrolysis when *T. pyriformis* is grown on casein at two different values of initial pH.

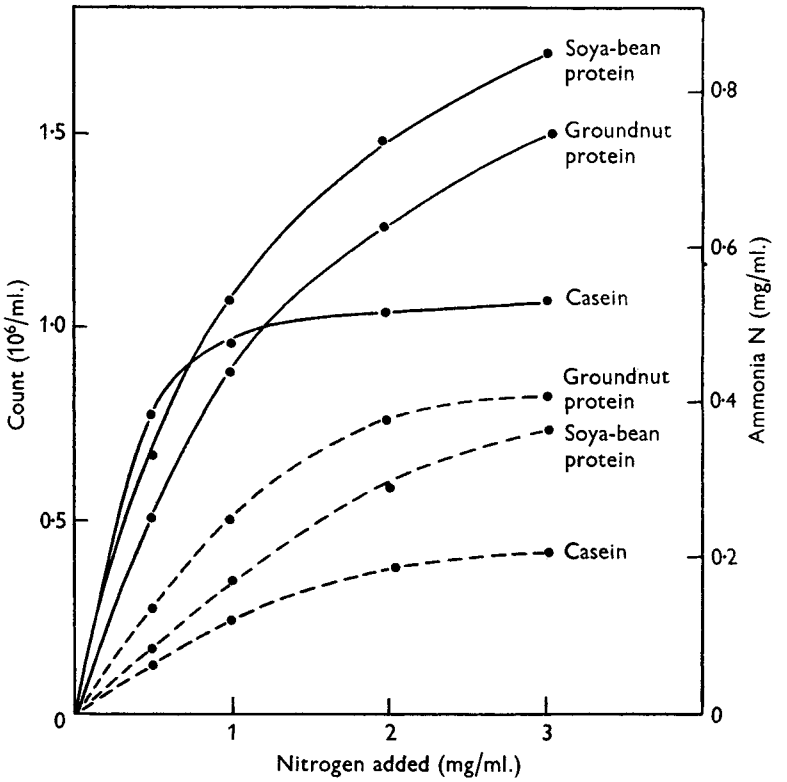

Fig. 3. Growth and ammonia production of *T. pyriformis* on 'vitamin-free' casein, groundnut and soya-bean proteins. ——, count; - - - - -, ammonia N.

Table 4. *Relationship between count and percentage hydrolysis with* Tetrahymena pyriformis *grown on 'vitamin-free' casein*

N added (mg/ml.)	pH	Count (10^6/ml.)	Hydrolysis (%)	Ratio, count:percentage hydrolysis
0·5	5·5	0·60	71	0·00845
0·5	7·5	0·78	94	0·00830
1·0	5·5	0·75	58	0·0129
1·0	7·5	1·02	78	0·0131
2·0	5·5	0·90	52	0·0173
2·0	7·5	1·05	64	0·0164

At a given N intake hydrolysis and growth were both restricted at the lower pH, but the relationship between them, expressed as a ratio of count to percentage hydrolysis, remained substantially the same. In attempting to relate nutritive values obtained with *T. pyriformis* to those for higher animals, a criterion based on growth alone is apparently not sufficient.

The end-product of N metabolism in *T. pyriformis* has been shown by Lwoff & Roukhelman (1926) and by Doyle & Harding (1937) to be mainly, if not exclusively, ammonia.

The amount of ammonia accumulating in the culture medium will reflect the amount of N metabolism occurring in protein synthesis, in tissue maintenance and in utilization of amino-acids for energy purposes and as a source of carbon. If the non-specific use of amino-acids as a primary source of energy and carbon can be restricted by the provision of adequate amounts of glucose, then the amount of ammonia formed will serve as an index of the extent of protein metabolism occurring in tissue synthesis and maintenance. The ratio of count (protein synthesis) to the ammonia N can then be taken to represent the efficiency of protein utilization and, for a given amount of tissue synthesis, a low-quality protein will yield relatively more ammonia than one of higher quality and a correspondingly lower count:ammonia N ratio.

Table 5. *Count:ammonia N ratios and relative nutritive values for* Tetrahymena pyriformis *of casein, groundnut and soya-bean proteins for the range* 0–3 *mg N/ml.*

Protein	Area under response curves (sq. in.)		Ratio, count:ammonia N	Relative nutritive value
	Count	Ammonia N		
Casein	27·1	8·4	3·23	100
Soya-bean	35·2	13·1	2·69	83
Groundnut	29·2	17·3	1·69	52

This criterion can be applied to the results on casein and on soya and groundnut proteins (see Fig. 3). Count and ammonia N responses were integrated for the range 0–3 mg/ml. by taking areas under the curves, and the ratio, count:ammonia N was calculated (Table 5). These ratios, expressed on a scale relative to casein taken as 100, constitute relative nutritive values (R.N.V.) for *T. pyriformis*.

The relative nutritive values obtained were in the order of their ratings for higher animals.

The effect of glucose on ammonia production. Before undertaking a survey of proteins it was necessary with the above-mentioned method of comparing nutritive values to examine in more detail the relationship between glucose in the medium and ammonia production.

Loeffer (1938) calculated that the ciliates utilize $0{\cdot}23-0{\cdot}40\times10^{-6}$ mg glucose/organism/h. Since *T. pyriformis* populations of up to 2×10^{6} organisms/ml. are obtained when grown on proteins at 3 mg N/ml., the glucose consumed per ml. over a 24 h period on this basis could be 10·3–19·2 mg, which would be equivalent to a glucose concentration of between 1 and 2%.

The sparing effect of glucose on ammonia production by *T. pyriformis* grown on casein is shown in Fig. 4. The reduction in ammonia was striking, amounting to one-half at the 2% level. Above 2% the effect diminished. The effect of glucose on growth was relatively small up to 5%, but concentrations above 5% led to a complete inhibition of growth.

The inclusion of high levels of glucose served two purposes—to restrict the utilization of amino-acids as energy and carbon sources and to minimize the effect of carbohydrate in the protein preparations on the count:ammonia N ratio. To avoid possible inhibition at 5%, a glucose concentration of 4% in the basal medium was adopted.

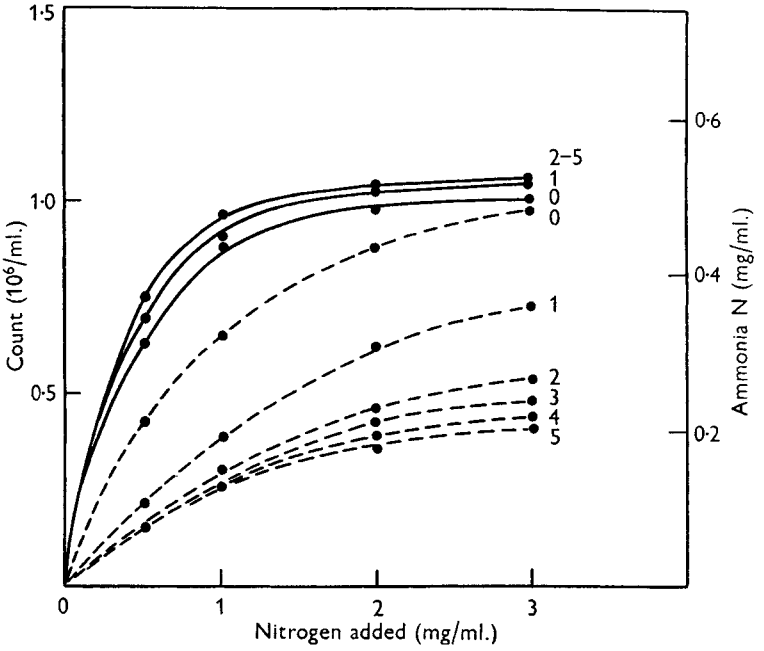

Fig. 4. Effect of glucose concentration on the growth and ammonia production of *T. pyriformis* on 'vitamin-free' casein. ——, count; - - - - -, ammonia N. The values at the ends of the curves give the percentage of glucose in the medium.

Amide N. Vegetable proteins are known to contain appreciable quantities of amide N, mainly as glutamine and asparagine. Glutamine was shown to undergo alkaline hydrolysis and yield ammonia when subjected to the distillation conditions employed for the determination of ammonia. When *T. pyriformis* was grown on vegetable proteins known to contain glutamine and the culture fluids were examined for free 'glutamine' N, no significant amounts were found. Presumably amide compounds such as glutamine and asparagine are deaminated by the ciliate and the resultant acids are utilized or excreted (Wu & Hogg, 1952).

Survey of proteins. Table 6 records the results of assays on a range of animal and vegetable proteins. Count:ammonia N ratios were obtained from the range 0–3 mg N/ml. for a 4-day growth period with a glucose concentration of 4%. Relative nutritive values were then expressed as a percentage of that of casein taken as 100.

The relative nutritive values obtained with *T. pyriformis* over the wide range of protein materials assayed are in general agreement with chemical scores and with biological assays on growing rats. In general, the vegetable proteins were inferior to those of animal origin. The high value for wheat germ and the very low rating for gelatin accord with observations on rats. Gelatin lacks the essential amino-acid

tryptophan, and the R.N.V. of 3 is not unexpected; the R.N.V. of gelatin supplemented with 2% tryptophan was 37. The loss in nutritive value of groundnut meal when subjected to excessive heating was demonstrated by the drop in R.N.V. from 51 to 31. Fresh egg white, sterilized by filtration, did not support the growth of *T. pyriformis*. Heating overcame this inhibition of growth and an R.N.V. of 115 was obtained. This phenomenon suggests a mechanism similar to the anti-tryptic behaviour of egg white in higher animals, to which notably the mouse and dog are particularly sensitive.

Table 6. *Relative nutritive values of partially purified proteins for* Tetrahymena pyriformis *compared with ratings by other methods (expressed on the basis, casein* = 100)

	Relative nutritive value	'Chemical score'*	Net protein utilization (rat) *	Net protein utilization (rat) †	Protein efficiency ratio (rat) ‡
Meat meal	132	—	—	—	—
Fish meal (oily)	130	—	—	—	—
Herring meal	130	—	—	—	—
Whale-meat meal	129	—	—	—	—
Egg albumen (commercial)	121	113	122	137	119
Wheat germ	115	71	105	112	—
Casein ('vitamin-free')	100	100	100	100	100
Soya-bean meal	83	87	106§	93	—
Cottonseed meal	81	64	78	98	—
Sunflower meal	74	90	90	—	—
Linseed cake	66	—	107	92	—
Soya-bean meal (overheated)	66	—	—	—	—
Sesame meal	53	67	96	—	—
Groundnut meal	51	45	82	71	51
Groundnut meal (overheated)	31	—	—	—	—
Gelatin	3	0	35	3	—
Gelatin + 2% tryptophan	37	—	—	—	—
Egg white (raw)‖	0	—	—	—	—
Egg white (autoclaved)	115	—	—	—	—

* Block & Mitchell (1946–7).
† Miller & Bender (1955).
‡ Rutgers University: Bureau of Biological Research (undated).
§ Highest value.
‖ Sterilized by filtration.

DISCUSSION

It is difficult to compare the populations achieved in our study with those of other workers on *T. pyriformis*, since few authors quote actual numbers of organisms. Nevertheless, the populations of *T. pyriformis* obtained were greater than those previously reported. This was due to the use of shallow cultures and the provision for highly aerobic conditions during growth. Under these conditions populations of 10^6 ciliates/ml. could be achieved on amino-acid media. For assay purposes on proteins the range 0-3 mg N/ml. has been used and populations up to 2×10^6/ml. have been obtained. When the organism was grown at higher levels, up to 6·0 mg N/ml., populations as high as 4×10^6 ml. have been observed.

Pace & Ireland (1945) found that with increasing pressures of oxygen a progressive increase in the growth was obtained. They also found that growth of *T. pyriformis*

ceased below an oxygen tension of 10 mm Hg and that the organism died at a carbon-dioxide tension above 120 mm Hg. The extent of the organism's requirement for oxygen can be gauged by considering the q_{O_2} of 225 (μl. gas/mg N/h) determined by Ryley (1952) on *T. pyriformis* in non-nutrient medium. The oxygen uptake for 1 mg of ciliate N (*c.* 5×10^6 organisms) over a period of 36 h would be over 8 ml. The oxygen demand of many cultures grown over 4 days might greatly exceed this amount.

The equivocal nature of the methods used by Rockland & Dunn (1949) and Pilcher & Williams (1954) to measure growth response has been discussed above. From both reports it is evident that the relative growth responses to various proteins altered as incubation proceeded, and that the time at which growth was assessed greatly affected the correlation with values obtained on higher animals. During our investigation a direct relationship between growth and accepted values of protein quality could not be obtained. This lack of relationship was considered to be due, at least in part, to the differing digestibilities of the proteins.

The adoption of the count:ammonia N ratio is an attempt to measure not the total amount of protein synthesized but the metabolic efficiency with which the synthesis of protein has been achieved. It is assumed that, in the presence of adequate amounts of glucose, ammonia as the main, and possibly the only, nitrogenous end-product of metabolism, will be related to the amount of protein metabolized. Lwoff & Roukhelman (1926) and Doyle & Harding (1937) found no evidence for the formation of urea as an end-product of N metabolism in *Tetrahymena*, though Nardone & Wilber (1950) have reported the presence of substantial amounts of urea in young cultures. In our work the presence of urea or 'glutamine' N could not be demonstrated in cultures grown on casein and nutrient broth examined over an incubation period of 4 days.

Even though good agreement has been obtained between protein quality assays with *T. pyriformis* and with the growing rat, it is difficult at this stage to assess the relationship of the criteria employed. The count:ammonia N ratio is an attempt to measure the efficiency with which protein, digested by the organism, is utilized in tissue synthesis, whereas protein efficiency ratio (the gain in body-weight (g) per g protein (N $\times$ 6·25) consumed) and net protein utilization (that fraction of N fed retained in the body) are based on the total N fed. Criteria used for higher animals that deal, not with total N ingested, but with N absorbed after digestion, such as N balance index of absorbed N (rate of change of N balance with respect to absorbed N (Allison, 1949)) and biological value (that fraction of absorbed N retained in the body), are in a sense more closely related to the *T. pyriformis* criterion. Nevertheless, the fundamental morphological and physiological differences between higher animals and the ciliate invalidate further attempts at correlation. The present inability to differentiate between hydrolytic and excretory products in *T. pyriformis* makes it doubtful whether analogous criteria can be developed.

The results of the survey of animal and vegetable proteins are in broad agreement with chemical scores and with nutritive values determined on higher animals. A reservation must be made about the actual values obtained in this survey. The relative nutritive values were determined on partially purified proteins, so as to minimize the influence of non-protein components. The protein assayed, therefore,

may not be representative of the total protein present in the parent material, owing to incomplete extraction by the procedure employed. The envisaged application of the method to the direct assay of foodstuffs raises the problem of the influence of non-protein components, such as minerals, vitamins, purines and pyrimidines and glucose, on the count:ammonia N ratio. This problem is considered by Rosen & Fernell (1956).

A feature revealed in the assay was the inhibition of growth by native egg albumen. The beneficial effect of heat on crystalline egg albumen has been observed by Viswanatha & Liener (1955), but whether the improvement of raw egg white on autoclaving is due to denaturation or to the destruction of anti-trypsin has not yet been determined. The response to amino-acid supplementation, illustrated by the effect of L-tryptophan on gelatin, not only increases confidence in the method, but also, in view of the widening interest in amino-acid supplementation of foods and feeds, extends the potentialities of the assay procedure.

SUMMARY

1. The effects of time, oxygen limitation, and glucose concentration on the growth and metabolism of *Tetrahymena pyriformis* W were studied.

2. It was demonstrated that highly aerobic conditions must be used for maximum growth.

3. A marked reduction in ammonia production occurred when high levels of glucose were included in the medium. This reduction is considered to be due to a sparing effect on the wasteful utilization of amino-acids as a primary energy source.

4. Growth by itself did not accord with accepted nutritional ratings of proteins. This disagreement is thought to be due to differences in hydrolysis, influenced by the nature and physical state of the protein.

5. Growth in relation to ammonia-nitrogen production is taken as an index of the efficiency of protein utilization. This criterion was used for the calculation of relative nutritive values.

6. The relative nutritive values of proteins for *T. pyriformis* were in general agreement with ratings for the growing rat.

7. Groundnut and soya-bean proteins from overheated meals showed lowered nutritive values.

8. The beneficial effect of tryptophan supplementation on gelatin was demonstrated.

9. Growth inhibitors in raw egg-white were inactivated by autoclaving.

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Microbiological evaluation of protein quality with *Tetrahymena pyriformis* W

2. Relative nutritive values of proteins in foodstuffs

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Conditions for the growth of *Tetrahymena pyriformis* W on intact proteins and the principle of a method for the determination of relative nutritive values have been described (Fernell & Rosen, 1956). The relative nutritive values of a number of partially purified proteins were in general accord with chemical scores and with protein ratings for the growing rat. These studies on the potentialities of *T. pyriformis* for the microbiological evaluation of proteins were continued with direct application of the method to foodstuffs, such as oilseed meals and animal-protein concentrates, without preliminary separation of the protein. Since relatively little was known about the physiology and biochemistry of ciliates cultured on intact proteins, the choice lay initially between a detailed investigation of one, or possibly two, protein sources, and a less detailed survey of a wide range of protein types in foodstuffs. We have tended to follow the latter course on the grounds that it might reveal limitations of the criterion adopted for comparing protein qualities.

In this second stage it was important not only to take into account the effect on the practical procedure of substantial quantities of non-protein material in foodstuffs, but