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Microbiological evaluation of protein quality with *Tetrahymena pyriformis* W

3.* A simplified assay procedure

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Methods for assessing the nutritive characteristics of proteins range from feeding trials using practical mixed diets with human and animal subjects to the laboratory evaluation of a single chemical property, such as the methionine content, of a single protein source. Between these two extremes there are, for example, rat and chick assays designed as biological screening tests for single protein sources. Biological tests with these species are relatively expensive and time-consuming, but nevertheless they do encompass digestibility and amino acid availability. The 'available lysine' test of Carpenter, Ellinger, Munro & Rolfe (1957) is so far the only purely chemical test designed to measure available as opposed to total amino acid content, and with the other amino acids the need for a biological test is becoming increasingly apparent.

Microbiological assays of intact proteins make it possible to do a large number of rapid and comparatively inexpensive biological assays, with due emphasis on the availability of essential amino acids. The scope and potentialities of such tests have been reviewed by Rosen (1959), Ford (1960) and Rosen, Stott & Smith (1960) and it is sufficient here to emphasize the double role of microbiological assays for measuring either the overall nutritive value of protein or the availability of individual essential amino acids.

Previous work demonstrated the ability of *Tetrahymena pyriformis* W to utilize a wide variety of protein sources, and it was shown that the relative nutritive values of the various types of protein source were generally similar to those for the growing rat (Fernell & Rosen, 1956; Rosen & Fernell, 1956). Modified techniques have since been applied to an extended range of materials and to the study of several aspects of the organism's metabolism and protein nutrition (Rosen *et al.* 1960). The measurement of organism response adopted by Rosen & Fernell (1956) was devised to determine the efficiency of protein utilization as expressed by the ratio of the number of organisms per ml culture medium to the amount of ammonia nitrogen present after 4 days' incubation. This approach was adopted because, under the chosen cultural conditions, a linear relationship between growth and protein level was not obtained. Later

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work on cereals and on protein concentrates damaged by heat (Rosen, 1959; Rosen *et al.* 1960) revealed that the ratio, count:ammonia N could give anomalous results under some conditions, and the possibility of using organism count as the criterion of nutritive value was considered. Further work confirming the value of the criterion and thus leading to simplification of the assay procedure is described in this paper.

EXPERIMENTAL

In previous work with 0·5 mg N/ml culture medium, the medium contained either 2 % starch or 0·75 % glucose. As a preliminary part of the work now described these two carbohydrates at these two levels were used in assays of fish meals and meat meals with N contents ranging from 0·1 to 0·5 mg N/ml culture medium. Later a study was made with media containing 0·75 % glucose and the 1·5 % originally used by Rosen & Fernell (1956) for assays with 1·0 mg N/ml.

To determine a suitable N level for assays in which growth (count) alone would suffice as the index of nutritive value, growth response of *T. pyriformis* W with meat meal, fish meal, dried whole egg, soya-bean meal, groundnut meal and wheat was measured over the range 0·1–0·5 mg N/ml assay medium.

In general the method followed that of Rosen & Fernell (1956) but, after the studies on the relationship of growth to N level had been completed, the measurement of ammonia N was omitted. The simplified assay procedure was:

(1) The test material was extracted three times with diethyl ether at room temperature and once with industrial methylated spirit (74 % over-proof). The material was finally washed with ether to remove residual alcohol and air-dried at room temperature. The dried material was then ground to pass a 72-mesh BS sieve. (The extraction and washing may be omitted for meals known to have been extracted with solvent.)

(2) The N content of the ground test material was determined by the Kjeldahl method, the digestion procedure of Chibnall, Rees & Williams (1943) and the steam-distillation procedure of Markham (1942) being used. The ammonia was distilled into 10 ml of 1 % (w/v) boric acid, and 0·1 N-HCl was used for titration with a methylene blue–methyl red indicator (Yuen & Pollard, 1953). Suspensions of test material were then prepared to give a N content of 1–5 mg/4 ml (3 mg/4 ml in routine assays) and the pH was adjusted to 8·2.

(3) Stock solutions A, B, C and D, all 100 times as strong as in the final medium, were prepared as shown in Table 1. Solution E, which was five times as strong as in the final medium, was then prepared from B, C, D and the appropriate weights of guanylic acid (sodium salt), adenosine-2′(3′)-phosphoric acid, cytidylic acid and uracil given in Table 1. This solution was adjusted to pH 8·2. In the earlier work (Fernell & Rosen, 1956) the pH used was 7·1, but 8·2 was now adopted to eliminate any tendency for the pH to drop below 7·1 when solution E plus protein suspension was autoclaved. With this modification the level of phosphate in the medium was reduced to that originally proposed by Fernell & Rosen (1956).

(4) Into each of a series of 2 oz 'medical flat' screw-capped bottles were pipetted:

(*a*) 2 ml of solution E; (*b*) 4 ml of 2·5 times strength test-material suspension; (*c*) 2 ml of distilled water (which volume may be used for the addition of supplementary amino acids). The bottles were then autoclaved at 121° for 10 min, the time being reduced from the 15 min used previously to minimize the possibility of thermal damage to amino acids.

Table 1. *Composition of stock solutions and solution E*

Stock solution A (100 times final strength)	(mg/200 ml)
Calcium pantothenate	12·5
Nicotinamide	12·5
Pyridoxine hydrochloride	125·0
Pyridoxal hydrochloride	12·5
Pyridoxamine hydrochloride	12·5
Riboflavin	12·5
Folic acid	1·25
Thiamine hydrochloride	125·0
Inositol	12·5
Choline chloride	125·0
p-Aminobenzoic acid	12·5
Biotin	1·25
DL-α-lipoic acid	0·4

Stock solution B (100 times final strength)	(g/200 ml)
$MgSO_4.7H_2O$	2·8
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1·25
$MnCl_2.4H_2O$	0·025
$ZnCl_2$	0·0025

Stock solution C (100 times final strength)	(mg/200 ml)
$CaCl_2.2H_2O$	600
$CuCl_2.2H_2O$	60
$FeCl_3.6H_2O$	15

Stock solution D (100 times final strength)	(g/200 ml)
KH_2PO_4	3·5
K_2HPO_4	3·5

Solution E (5 times final strength)
(The appropriate weights of the components listed below are dissolved in approximately 10 ml distilled water, 1 ml stock solutions B, C, D added and the whole made up to 20 ml)

	mg/20 ml solution E
Guanylic acid (sodium salt)	15
Adenosine-2′(3′)-phosphoric acid monohydrate	10
Cytidylic acid	12·5
Uracil	5

(5) Two solutions were autoclaved separately at 121° for 10 min: (*a*) the stock vitamin solution A (Table 1) diluted ten times; (*b*) a solution containing 15% (w/v) glucose. This concentration of glucose was ten times that in the final medium.

(6) To each bottle after cooling were added aseptically 1 ml diluted solution A and 1 ml glucose solution.

(7) Assays were made with three replicates for each meal.

(8) Each bottle was inoculated with three drops of a 3-day broth culture of *T. pyriformis* W, and incubated at 25° for 4 days. The screw caps were left loose during this period and the bottles were inclined at 15° to the horizontal to provide sufficient aeration for rapid growth.

(9) After 4 days' incubation, the cultures were shaken for 2 min on a flask shaker and 1 ml of culture was transferred to a $\frac{1}{4}$ oz screw-capped bottle containing 1 ml preserving fluid (consisting of 90 ml water, 20 ml 36% (w/v) formaldehyde and 10 ml of stock solution D).

(10) Organisms were counted in a single-cell haemocytometer with Fuchs–Rosenthal ruling to BS 748. The organisms in eight alternate 1 mm squares were counted and the mean number per 1 mm square gave the final population of the test culture in units of 10^4 organisms/ml. This figure was used to express the nutritive value of the protein in test materials.

RESULTS AND DISCUSSION

Choice of carbohydrate source

Comparison of 2% starch and 0·75% glucose as carbohydrate sources over the range 0·1–0·5 mg N/ml culture medium showed that glucose usually gave a higher count figure than starch, as exemplified in Fig. 1. The use of starch also increased the opacity of the medium and thus rendered counting of the organisms somewhat more difficult than in the presence of glucose. These factors led to the adoption of glucose for routine assays. The further comparison of 0·75 and 1·5% glucose over the range 0·1–0·5 mg N/ml showed no difference in count, and 1·5% was therefore selected to ensure an ample carbon and energy supply in the basal medium.

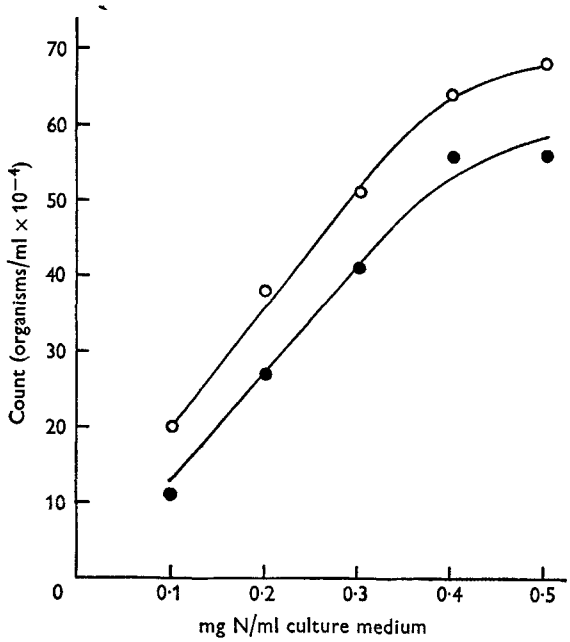

Fig. 1. Growth response of *T. pyriformis* W at low nitrogen levels, with glucose or starch as the energy source. ○, 0·75% glucose; ●, 2% starch.

In contrast to assays of simple protein sources, assays of mixed feeds, such as broiler, turkey and pig feeds, alone or in the presence of added starch or glucose, gave similar count values, indicating that the carbohydrate in the feed was itself sufficient for the energy requirements of the organism. However, since some mixed feeds intended for young animals, notably pigs and calves, may contain an appreciable proportion of lactose, which is not utilized by *T. pyriformis* W (unpublished findings), we have adopted 1·5% glucose for routine assays of animal feeds.

Growth response at low N levels

The growth response of *T. pyriformis* W with fish meal, meat meal, dried whole egg, soya-bean meal, groundnut meal and wheat is shown in Fig. 2, the sample code

numbers being those adopted for a collaborative investigation organized by the Agricultural Research Council (Boyne, Carpenter & Woodham, 1961). It will be seen that a straight-line response was obtained between 0·1 and 0·4 mg N/ml, which indicates that up to this point the growth of the organism was linearly related to the

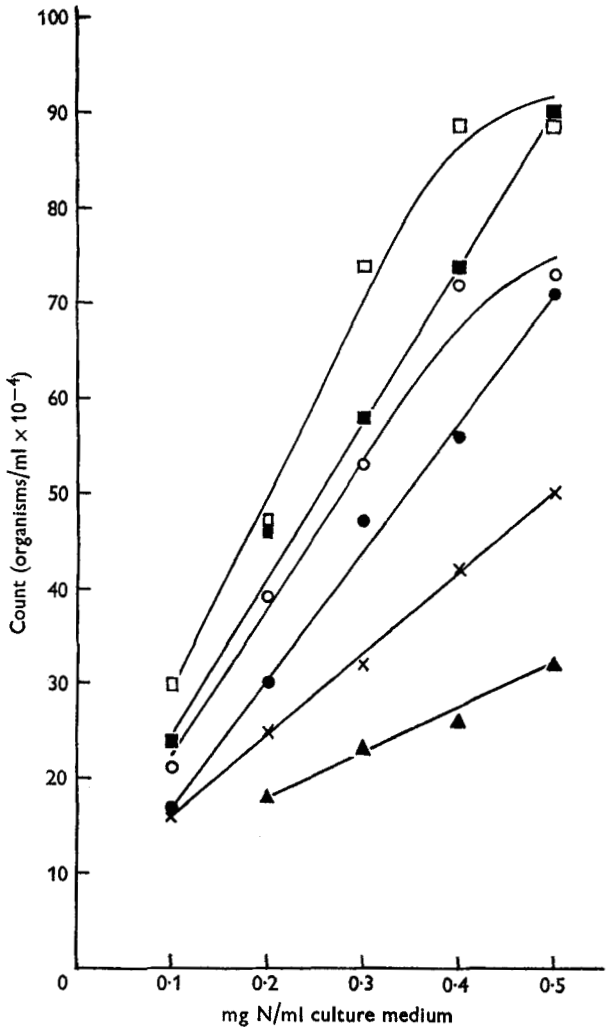

Fig. 2. Growth response of *T. pyriformis* W at low nitrogen levels to various protein sources. □, egg; ■, soya-bean meal SB1*; ○, fish meal FM2*; ●, meat meal MM1*; ×, groundnut meal GN5*; ▲, wheat.

*Sample code number used by Boyne *et al.* 1961.

amount of protein present. A concentration of 0·3 mg N/ml culture medium was chosen as a level at which the number of organisms present after 4 days' incubation would provide a suitable routine measure of the nutritive value of proteins.

The response curves in Fig 2 show similar nutritive values for the various protein sources over the range 0·1–0·4 mg N/ml, with the exception of the curves for groundnut meal and wheat, in which there is some suggestion of an influence of N level on

nutritive value. However, the count values for groundnut meal at 0·1 mg N/ml and for wheat at 0·2 mg N/ml are so low that an error of as little as one organism per 1 mm square of the counting chamber alters the apparent nutritive value by some 6 %, and it is thus quite probable that the divergence of nutritive values for groundnut and wheat over the N levels used is not significant.

Survey of protein sources

A wide range of protein sources has now been assayed with *T. pyriformis* W to determine the nutritive value with the simpler criterion of organism count. The range of values obtained in assays at 0·3 mg N/ml for various types of protein sources is given in Table 2. Results for some of the samples examined as part of the collaborative study already mentioned (Boyne *et al.* 1961) are included.

Table 2. *Nutritive value of protein in various sources determined with* Tetrahymena pyriformis *W, with a concentration of* 0·3 *mg nitrogen/ml culture medium and with* 1·5 % *glucose in the medium*

Protein source	Count (organisms/ml × 10^{-4})
Egg	77 (1)
Casein	59–66 (2)
Yeast	38–51 (2)
Meat meal	28–62 (14)
Fish meal	15–64 (35)
Whale-meat meal	26–38 (4)
Soya-bean meal	49–68 (11)
Groundnut meal	34–46 (11)
Cottonseed meal	29–32 (2)
Sunflower-seed meal	29–36 (3)
Wheat	20–36 (3)
Maize	18–22 (2)
Barley	16–29 (3)

Figures in parentheses are the number of samples assayed.

It will be seen that *T. pyriformis* assays graded egg as the best protein source, with casein and the best soya-bean, fish and meat meals of equal value farther down the scale. But it is significant that some animal protein sources were inferior to the worst samples of vegetable protein concentrates. Also there are whale-meat meals of higher nutritive value than those used in this particular study (Rosen *et al.* 1960). In general, fish meals and meat meals showed a wider range of nutritive value than the soya-bean and groundnut meals. The greater uniformity of these vegetable protein sources may derive in part from a lesser variation in the raw materials used in their manufacture and in part also from research on the technology of oilseed processing, which has enabled manufacturers to minimize damage by heat during processing.

Boyne *et al.* (1961), considering thirteen of the results for meat meals included in Table 2, noted that nutritive values determined with *T. pyriformis* showed a correlation with available lysine values (Carpenter *et al.* 1957) which was significant at the 5 % level, and also noted a correlation with gross protein values (Heiman, Carver & Cook, 1939) determined by chick-feeding tests. *T. pyriformis* results for eighteen

fish meals reviewed by the same authors showed no correlation with available lysine values, presumably because lysine was not the limiting amino acid for *T. pyriformis* in these meals.

Supplementation of protein sources by amino acids evoked various responses from *T. pyriformis* with the simplified procedure. The count value for wheat was increased from 24 to 53 by addition of 1% L-lysine. Barley, oats and rice also gave increased growth with added lysine, but maize and soya-bean did not. Soya-bean meal showed the expected methionine deficiency, with a small increase in organism count from 55 to 62 on addition of 2·5% L-methionine. Also, after a soya-bean meal had been heated for $1\frac{1}{2}$ h at 121°, assays showed no increase in growth on addition of lysine or methionine separately, but growth did increase with the addition of the two amino acids together. Further heating for 1 h at 121° resulted in lysine alone becoming the limiting amino acid in the meal.

SUMMARY

1. A simplified procedure for assay of the nutritive value of proteins with *Tetrahymena pyriformis* W is described.

2. The organism count after 4 days' incubation was used as a simple criterion of the nutritive value of protein.

3. Modifications of the cultural conditions, compared with those used by Rosen & Fernell (1956), were a reduction in the amount of phosphate in the basal medium and adjustment of meal suspension to pH 8·2 before autoclaving.

4. For assays of protein concentrates and mixed animal feeding-stuffs the nitrogen level in the medium was reduced to 0·3 mg/ml.

5. Glucose was found to be a more suitable energy source for routine assays than starch.

6. Results of assays on ninety-three protein foodstuffs illustrated the wide variation in nutritive value of individual types, especially fish meals and meat meals.

7. Typical improvements in the nutritive value of certain vegetable proteins when supplemented with lysine and methionine were observed with the simplified procedure.

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