# A Microbiological Approach to Nutritional Evaluation of Proteins. (20181)

SAM HALEVY* AND NATHAN GROSSOWICZ.

*From the Laboratory of Nutrition and Department of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel.*

The accepted methods for the determination of the nutritional value of proteins are based on a variety of physiological responses of common laboratory animals (mice, rats, dogs, etc.). All these assays are time-consuming, laborious and quite expensive, hence, that attempts are being made to devise more convenient methods. Melnick and Oser(1) showed that the rate of digestion of proteins with pancreatin, as measured by formal titration, is influenced by heat processing of the protein. This correlation was the basis of an *in vitro* method for estimating susceptibility of proteins to enzymic digestion. Anderson and Williams(2) used the growth response of the proteolytic ciliate, *Tetrahymena geleii*, towards different proteins as a measure of their biological value. These workers succeeded in overcoming certain technical difficulties encountered by earlier investigators (3,4) by incorporating 2,3,5-triphenyltetrazolium chloride into the medium. This compound is reduced by the protozoan to the red triphenylformazan, and is suitable for colorimetric determination despite the turbidity of the protein suspension.

It occurred to us that digestion of proteins with pancreatic enzymes, would yield hydrolysates which could be tested for their growth-promoting activity on a suitable test organism in a medium devoid of the 10 essential amino acids. The growth response would thus be determined in a single assay by the particular amino acid which appears in the mixture in a limiting concentration. Pancreatin, consisting of a mixture of most of the enzymes involved in the decomposition of proteins by higher animals and man, seemed a suitable digestive agent.

*Experimental. Procedure.* The method is carried out in 4 stages: a) The protein suspension is hydrolyzed with pancreatin. b) The growth-promoting activity (biological value?) of the hydrolysate on *Streptococcus faecalis*, in a medium devoid of the 10 essential amino acids, is tested; the growth response, in a single run, is determined by that essential amino acid which is present in limiting concentration. c&d) The actual composition of the hydrolysate with respect to the essential amino acids is determined and the effect of supplementing the limiting amino acids is analyzed. *Hydrolysis.* The enzymatic hydrolysis is carried out according to Melnick and Oser(1). To allow better contact between enzyme and substrate the digestion was carried out under constant shaking by means of a magnetic stirrer. Five g of a protein sample is suspended in 150 ml of alkaline buffer (pH 8.4), containing 300 mg of 3 U.S.P. Pancreatin and covered with a layer of toluene. The digestion is carried out for 48 hours at 37°C under constant shaking. About 40% of the protein was hydrolyzed by this treatment. Then, the remaining protein is precipitated by addition of glacial acetic acid and boiling for a few minutes. The supernate is filtered, the pH adjusted to 7.0, and the amount of the amino-N liberated is determined by the formal titration procedure. The solution is finally diluted to contain 1.4 mg of amino N per ml. *Composition of the medium.* The basal medium was prepared according to Henderson and Snell(5), the only important difference being the omission of the essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine). The pancreatic hydrolysates of the different proteins under test serve as the source for the essential amino acids. The original hydrolysate solution (1.4 mg amino N/ml was diluted till a growth response of about 40 to 50% of that of a control was obtained. The controls represent the growth of the test organism in the complete medium of Henderson and Snell(5) without any protein

* Based on data submitted in partial fulfillment of the requirements for the degree of Ph.D.

TABLE I. Comparative Growth of *Streptococcus faecalis* in Pancreatin Hydrolysed Proteins.

| | Amino N. γ/ml | Klett reading filter No. 660 | Relative activity casein = 100% | Rat growth test(6) casein = 100% |
|---|---|---|---|---|
| Egg albumin | 17.5 | 100* | 105 | 119 |
| Casein | 17.5 | 95 | 100 | 100 |
| Gelatin | 60.0 | 90 | 29 | — |
| Gluten | 87.5 | 70 | 15 | 16 |
| Zein | 175.0 | 20 | 2 | — |

* Tests were carried out in the Henderson Snell medium(5), from which the 10 essential amino acids were omitted and one of the protein hydrolysates incorporated. Growth of test organism in the complete medium(5) gives a turbidity reading of 220-250.

hydrolysate added.

*Test organism.* The organism studied most extensively was a strain of *Streptococcus faecalis*†) which requires all 10 essential amino acids. By arranging the experiment as described, a quantitative evaluation of the nutritive value of the hydrolysate for the given organism is thus obtained. If the protein tested is low in even one of the essential amino acids, or if enzyme treatment fails to liberate enough of an essential amino acid—its growth promoting activity will be low in comparison to others. *Estimation of growth.* The bacteria were grown in regular test tubes containing 2 ml of medium. The bacterial growth turbidity was estimated photometrically using the Klett-Summerson electrophotometer, after incubating the tubes at 37°C for 20-24 hours. The usual growth of the *S. faecalis* in the complete medium gave a reading of about 250, using the No. 660 filter. In cases where rather concentrated solutions of protein hydrolysates, *e.g.,* zein, had to be used, interference in the turbidity reading due to color of the hydrolysate was corrected by adjusting the zero point against an uninoculated tube of a similar sample. Other methods for estimating the bacterial growth may be used as well.

*Results.* Table I shows the comparative growth response of *S. faecalis* towards pancreatic hydrolysates of 5 proteins. Coagulated egg albumin and gelatin (Difco Laboratories, Inc.), casein and gluten (Nutritional Biochemicals Corporation), and zein (Bios Laboratories, Inc.) were used. As mentioned, this type of experiment was carried out by addition of different quantities of pancreatin treated protein to a medium devoid of the 10 essential amino acids, and the growth compared to that obtained in a complete medium of Henderson and Snell. The protein hydrolysate is usually diluted to give only a 40-50% growth as compared to a control on a complete medium. The readings may thus be made on the steep part of the growth curve and permit a more accurate evaluation of the results. In cases where still lower growth was obtained (a response of 10-30%), the results were interpolated from the curve. The test organism grows almost equally well on both casein and egg albumin (Table I). Gelatin, however, was much less active, the response being about 30% of that of casein. Gluten was even inferior to gelatin; the activity as compared to casein was about 15%. Zein was still less active and at high concentrations the growth was very small; the calculated activity was only about 2% as compared to casein.

A comparison of the results obtained with those given by the rat growth method(6) is shown in the last column of Table I. It will be noted that the agreement between the two methods is very close.

The next question to be studied was the nutritional basis for the results obtained namely which was the amino acid(s) limiting growth in the various protein hydrolysates. In order to obtain this information the deficient medium was supplemented with the usual amounts(5) of only 9 out of the 10 essential amino acids. A different amino acid was omitted in each run. The amount of protein hydrolysate remained as stated above. For the determination of the content of lysine, however, the amounts of both arginine and threonine were reduced from 200 γ per tube

† This strain was obtained through the courtesy of Dr. B. Volcani, The Weizmann Institute of Science, Rehovoth, Israel.

TABLE II. Effect of Supplementation with Essential Amino Acids on the Growth Response to Pancreatin Treated Proteins.*

| Deficient medium | Egg albumin, 17.5 γ/ml amino N A | Casein, 17.5 γ/ml amino N B | Gelatin, 60 γ/ml amino N C | Gluten, 87.5 γ/ml amino N D | Zein, 175 γ/ml amino N E | Gluten‖ heated, 115 γ/ml amino N F | Gluten‖ heated with glucose, 175 γ/ml amino N G |
|---|---|---|---|---|---|---|---|
| a. 10 essential amino acids missing | 100† | 95† | 95‡ | 70† | 20† | 100§ | 30§ |
| b. Plus 9 essential amino acids Amino acid omitted: | | | | | | | |
| Arginine | 150 | 145 | 195 | 270 | 250 | 190 | 170 |
| Histidine | 150 | 240 | 200 | 260 | 240 | 210 | 165 |
| Isoleucine | 180 | 185 | 170 | 260 | 240 | 210 | 170 |
| Leucine | 210 | 250 | 155 | 260 | 240 | 210 | 165 |
| Lysine | 125 | 135 | 190 | 65 | 15 | 95 | 35 |
| Methionine | 250 | 240 | 165 | 260 | 230 | 220 | 175 |
| Phenyl alanine | 240 | 240 | 195 | 240 | 230 | 190 | 170 |
| Threonine | 165 | 180 | 165 | 260 | 230 | 185 | 165 |
| Tryptophane | 250 | 240 | 105 | 260 | 250 | 210 | 175 |
| Valine | 210 | 210 | 200 | 260 | 240 | 185 | 165 |
| c. Supplemented with: | | | | | | | |
| Arginine, histidine, isoleucine, lysine, and threonine | 240 | | | | | | |
| Arginine, isoleucine, lysine, and threonine | | 250 | | | | | |
| Lysine | | | | 270 | 260 | | |
| Tryptophane | | | 165 | | | | |
| Isoleucine, leucine, methionine, threonine, and tryptophane | | | 210 | | | | |

* Test organism used: *S. faecalis*. Numbers represent Klett turbidity readings. Growth in complete Henderson-Snell medium (without protein hydrolysate added) was: † 250; ‡ 225; § 210.

‖ Samples treated for 2 hr at 15 lb pressure before enzymatic digestion.

to 20 γ per tube. These changes were introduced in order to avoid inhibition of the utilization of the lysine of the hydrolysate.‡

It is evident that the limiting factor in the egg albumin preparation is lysine (Table II-A). When all amino acids except lysine were added to the protein hydrolysate, growth increased only slightly (from 100 to 125) on the turbidity scale. Addition of lysine alone did not restore full growth. Here arginine and histidine became limiting (the reading was 150 in the absence of either arginine or histidine). Other amino acids appearing in limited concentration were threonine, isoleucine, leucine and valine. Tryptophane and methionine were found in excess; the omission of either does not affect the full response. Supplementation of the egg albumin sample with the missing amino acids (Table II-A, c) brings up the growth to that of the control (Table II, footnote 2). Column B shows similar results for casein. In this case the number of limiting amino acids is restricted to lysine, arginine, isoleucine and threonine, the first two being the more important ones. Using gelatin, the main limiting amino acid was tryptophane (Column C). Supplementation with tryptophane alone (C, c) was insufficient to secure full growth, as leucine, isoleucine, methionine and threonine were also limiting. The low activity of gluten was due to the low content of lysine; supplementation with lysine alone resulted in a full response (Column D). Essentially the same is true for zein (Column E).

*Effect of heating with glucose.* Heating the protein samples in presence of glucose before

‡ Grossowicz, N., and Halevy, S., unpublished data.

enzymic hydrolysis reduces markedly the growth of *S. faecalis*. A sample containing 6 g of gluten and 5 g of glucose was moistened with 2.5 ml of water and autoclaved for 2 hours at 15 lb pressure. A control sample without glucose was similarly treated. The growth responses to the untreated versus the glucose heated samples of gluten, are shown in columns F and G, respectively (Table II). It will be noted that heating with glucose prior to enzymatic treatment reduced the response to 20%. This deleterious change was due to the reduction in available lysine; the concentration of all the other amino acids became limiting, too, but to a lesser extent.

*Discussion.* Chemical hydrolysis of proteins with strong acid or alkali provides a mixture of amino acids the composition of which has been estimated by numerous investigators quite accurately using microbiological, chemical, or physical methods. The methods provide, at the best, data from which the theoretical composition of amino acids of various proteins could be estimated. None of them, however, give any clear idea of the nutritional value of the proteins in question. For this purpose various kinds of biological assays are being used. The reason for using these methods (growth or gain of weight of young animals, maintenance in adult animals, nitrogen balance tests, etc.) is that the biological value of a given protein depends not only on its composition in amino acids, but, among other factors, also on the extent of the liberation of the different amino acids during the process of digestion (enzymatic hydrolysis). In the body not only the composition, but also the physical state of the protein, for example whether heated or not, plays an important role. The idea was therefore advanced that treatment of proteins with a mixture of the enzymes normally active for this purpose in the body in the form of pancreatin, would provide an hydrolysate containing the essential amino acids in their "biological ratio," capable of being assayed microbiologically. A correlation, however, with one of the common biological tests would depend on whether or not a suitable organism was found.

The results presented (Table I), although based on a few samples only, show such a correlation for 3 of the 5 proteins tested. There is good agreement for casein, egg-albumin and gluten between results obtained by the rat growth method(6) and the pancreatin treated samples as assayed with *S. faecalis*. As for the 2 remaining proteins, the low value for zein seems correct, although comparative data are missing. The results for gelatin, however, appear too high in comparison with the rat growth test(7).

Our tests showed that the low biological value of both gluten and zein were due to low lysine, while gelatin was a poor protein on account of being limiting in tryptophane—findings which are in accordance with those obtained by other methods(7). A few discrepancies were also found while the nutritional value of egg-albumin and casein rated as high as in the rat growth test(6), both proteins were found to be deficient in lysine by our test. On the other hand, according to other methods, the limiting factor in egg-albumin is isoleucine and in casein it is methionine(8). A possible explanation for the differences obtained with our method may be due to an exaggerated requirement of lysine by *S. faecalis* in comparison to the rat. Another possibility, however, should not be overlooked. The rate of utilization of a given amino acid may depend on its quantitative relationship or balance towards the other amino acids present. Excess of arginine or threonine or both may inhibit markedly the utilization of lysine present in casein and egg-albumin hydrolysates.‡ The method appears, therefore, very suitable for studying imbalances among amino acids and their effects upon the nutritional value of the different proteins.

The nutritive value of a given protein may vary according to the criteria employed; using the maintenance method, wheat gluten appears to be as good a protein as beef muscle, while for growth the latter is definitely superior(9).

Whereas our results seem to agree with those obtained by the rat growth method(6), it appears possible that other micro organisms may follow the patterns obtained with some of the other biological methods (maintenance,

repletion, etc.). This possibility is now being investigated.

*Summary.* 1. A microbiological method to assess the biological value of proteins is described. It implies the digestion of proteins with pancreatin and subsequent testing of the growth-promoting activity of the hydrolysate for *S. faecalis*, in a medium devoid of the 10 essential amino acids. The nutritive value of the protein digest is determined in a single assay by the amino acid liberated in a limiting concentration. 2. The results obtained with casein, egg albumin, gelatin, gluten, and zein are presented and their agreement with the biological values obtained by the rat growth method is suggested. 3. The growth promoting activity (biological valued) of casein and egg albumin is high and about equal; gelatin and gluten, in comparison, are much less active, 29 and 15%, respectively, and zein still less (2%). 4. At the limited growth level, casein is deficient in: lysine, arginine, isoleucine, and threonine. Egg albumin under comparable conditions is limited in lysine, arginine, histidine, threonine, isoleucine, leucine, and valine. Gelatin is low in tryptophane, isoleucine, leucine, methionine, and threonine. Gluten and zein are short in lysine. 5. The suitability of the method described for studying imbalances of amino acids is proposed.

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Received February 11, 1953. P.S.E.B.M., 1953, v82.

## Induction of Diabetes Insipidus in Adrenalectomized Dogs with Cortisone.* (20182)

W. W. SWINGLE, E. J. FEDOR, MAX BEN, ROBERT MAXWELL, CARLETON BAKER, AND GEORGE BARLOW.

*From the Biological Laboratory, Princeton University, Princeton, N. J.*

Large daily doses of DCA, when administered to intact dogs over a sufficiently prolonged interval, induce a syndrome of polyuria and polydipsia apparently identical with that of diabetes insipidus. According to Ragan *et al.*(1), pituitrin is relatively ineffective in controlling the polyuria and fluid restriction does not cause dehydration. Mulinos *et al.*(2) observed that withdrawal of salts from the diet reduced the severity of the polyuria and that large doses of pitressin in oil led to reduction of water intake and urine volume.

Recently it has been shown that daily injections of cortisone given to normal dogs for periods ranging from 2-3 weeks also bring about a marked polyuria and polydipsia(3,4). Sirek and Best(4) report that 10 I.U. of posterior pituitary hormone administered to one dog for one day restored almost normal conditions so far as thirst, polyuria and urine specific gravity were concerned.

*Material and methods.* Four mongrel male dogs were used in the present experiments; they had been adrenalectomized for periods varying from 8 months to 2 years. The animals were kept in metabolism cages and fed a special diet consisting of Warner's dog food,

* A part of the expenses of this investigation was defrayed by a grant from Sharp & Dohme, West Point, Pa.