Technical Papers

Deposition of Calcium Phosphates Accompanying Senile Degeneration and Disease

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The substance of certain pathological calcareous concretions or plaques variously found in the lachrymal duct, prostate gland, and the walls of bronchial tubes and arteries has been loosely described as "phosphate and carbonate of lime," "earth of bones," "earthy phosphate mixed with carbonate of lime," and, more generally, simply as calcium phosphate, ever since the time of the first description by Wollaston in 1797. The question whether the carbon dioxide found on the chemical analysis of such materials is present as admixed carbonate or as an integral part of a single phase has been uncertain. Investigations in recent years by X-ray diffraction and optical methods have failed to show admixed calcium carbonate and phosphate in bone and teeth, however, and it is now generally held that these deposits consist of a substance with an apatite-type structure that contains constitutive carbonate ion. It may be noted that tricalcium phosphate hydrate, $Ca_3(PO_4)_2 \cdot nH_2O$, gives an X-ray powder diffraction pattern similar to that of apatite and is isomorphous therewith. Carbonate can enter this compound, as in apatite. The material can be distinguished (*2*) from hydroxylapatite, $Ca_{10}(PO_4)_6(OH)_2$, by heating to *ca.* 900° C., where it breaks down to β-$Ca_3(PO_4)_2$, whitlockite, and then gives the X-ray pattern of that substance. On the other hand, apatite, specifically including carbonate-apatite, and whitlockite give an unchanged pattern after such treatment.

By chemical, optical, and X-ray studies we have identified carbonate-apatite as the principal inorganic salt in the pathological calcification of tuberculous lymph nodes and subcutaneous hematomata. A concretion obtained from a chronically infected prostate gland was found to be composed of fine-grained carbonate-apatite together with crystals of struvite, $NH_4MgPO_4 \cdot 6H_2O$, and an unidentified substance. Brushite, $CaHPO_4 \cdot 2H_2O$, also has been reported in prostatic deposits. Calculi removed surgically from the sublingual salivary glands in several patients were identified as carbonate-apatite. We have also found that the common plaques developed in arteriosclerosis of the aorta and other large arteries, composed largely of cholesterol, frequently contain a small amount of carbonate-apatite. The occurrence of an apatite-like substance was earlier noted by Saupe and Klötzer (*5*) in aortal concretions and by Huggins and Bear (*4*) in prostatic calculi. The "calcium phosphate and carbonate" reported in tonsillar and pancreatic calcifications probably is carbonate-apatite. The latter has also been reported as a common constituent of calculi in the kidneys and urinary bladder by Frondel and Prien (*1*).

A variety of whitlockite containing a small amount of carbonate and analogous to carbonate-apatite was identified as composing a small pathological calcification of the appendix testis or hydatid of Morgagni. This substance also has been found as calculi in the urinary tract. We have not found tricalcium phosphate hydrate in any pathological calcification so far examined, although Hendricks and Hill (*3*) point out that the available evidence indicates that it is the principal constituent of bone.

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Rate of Enzymic Digestion of Proteins as a Factor in Nutrition[1]

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The principal factors which determine the value of a food as a source of protein are (1) its protein content in relation to total solids, (2) the essential amino acid composition of the protein, and (3) the availability of these essential amino acids for tissue protein anabolism. It has been found (*5*) in studies with protein hydrolysates that the omission of a single essential amino acid from an otherwise adequate mixture results in poor utilization of the absorbed

[1] The material in this report constitutes part of a paper presented by one of us (B.L.O.) before the Section on Food and Nutrition, Gibson Island Conference of the American Association for the Advancement of Science, July, 1945.

amino acids. If the missing amino acid is injected separately eight hours after the basic mixture is administered, the retention of amino acid nitrogen is not improved. The working hypothesis in the present study states that for optimum utilization of food proteins all essential amino acids must not only be available for absorption but must also be liberated during digestion *in vivo* at rates permitting mutual supplementation.

Of the various methods for biologically estimating the nutritive value of proteins, one of the best is the nitrogen balance method of Mitchell (*11*). This procedure demonstrates, but does not explain, why a given protein may show differences in biological value after heat treatment even though its ultimate amino acid composition and degree of digestibility remain unchanged. By supplementing the bio-assay technic with the *in vitro* digestibility procedure described in this report, the probable basis for this phenomenon can be elucidated.

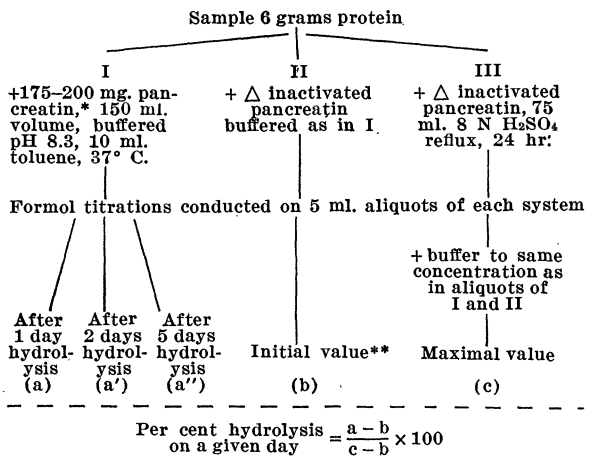

FIG. 1. *In vitro* technic for estimating susceptibility of proteins to enzymic digestion.
* Quantity of U.S.P. pancreatin dependent upon potency of preparation.
** Includes the titration of the free amino groups in the intact proteins plus the titration of the buffer.

The *in vitro* method involves periodic measurements of the degree of hydrolysis of the protein by a modified formol titration procedure. The details of the method are presented in Fig. 1. The use of suboptimal quantities of pancreatin permits digestion to proceed for several days at 37° C., thus retarding the liberation of the amino acids to reveal differences in rate of digestibility.

Details of the formol titration conditions will be reported elsewhere. Suffice it to say that the titrations were terminated at pH 9.5, instead of pH 8 to 9, yielding values for formol titratable nitrogen in closer agreement with total nitrogen; pancreatic activity was standardized; naturally present enzymes were inactivated and bacterial action prevented; and finally, amide nitrogen and fat were not interfering factors under the conditions employed.

Correlation of the results obtained by this empirical *in vitro* test with more than 30 biological assays of five different sources of food protein has emphasized the value of supplementing the biological data with evidence on the susceptibility of these proteins to enzymic digestion. The animal experiments involved both nitrogen balance studies with the rat and growth studies with both the rat and the chick.

Soybean protein is especially suited to the study of the above-stated hypothesis, since it shows no change in amino acid composition or in degree of digestibility before and after various types of processing, but exhibits marked differences in biological value (retention of absorbed nitrogen).

The data in Table 1 show that those factors known (*12*) to increase the nutritive value of the soy protein also affect the susceptibility of the protein to enzymic

TABLE 1
SUSCEPTIBILITY OF SOY PROTEINS TO ENZYMIC DIGESTION

Sample (protein content)	Laboratory processing	Hydrolysis of proteins		
		After 1 day	After 2 days	After 5 days
		Per cent		
Solvent-processed soy flour (52.7%)	None	8	9	11
	100° C., 7 days, atmospheric pressure	8	9	11
	in water, 100° C., 5 min.	7	9	11
	Autoclaved 10 min. at 5 lbs. pressure	9	10	14
	Autoclaved 10 min. at 10 lbs. pressure	12	21	24
	Autoclaved 30 min. at 10 lbs. pressure	14	23	29
Expeller-processed soy grits (48.7%)	None	16	20	24
	Autoclaved 10 min. at 10 lbs. pressure	20	23	29
	Autoclaved 30 min. at 10 lbs. pressure	22	27	33
	Autoclaved 30 min. at 15 lbs. pressure	18	25	34
	Autoclaved 45 min. at 15 lbs. pressure	16	25	33

digestion. Dry heating of soy flour at 100° C. at atmospheric pressure, or heating the meal in boiling water, induces no improvement in the biological value of its protein. However, autoclaving causes a pronounced increase. It will be noted that with more effective autoclaving treatments the susceptibility of the soy protein to enzymic digestion increased progressively until a plateau in values was reached. Improvement in biological value due to the method of manufacturing soy grits was evidenced by the greater degree and rate of digestibility of the original material.

Almquist and collaborators (*2*) and Hayward and Hafner (*8*) demonstrated that the limiting amino acid

in soy protein is methionine and that heating under pressure increases the availability of this amino acid. The fact that it is the rate rather than the extent of liberation of the methionine in soy protein which determines the biological value of soy protein is evident from the illustrative data in Table 2. Using the nitrogen balance technic described by Mitchell (*11*),

TABLE 2

RATE OF ENZYMIC LIBERATION OF AMINO ACIDS DURING THE *in Vitro* TEST OF PROTEIN DIGESTIBILITY AS A CRITICAL FACTOR IN DETERMINING NUTRITIONAL RESPONSE

Experiment	Raw soy meal	Processed soy meal
Rat assays		
Biological value of the proteins, per cent	53	71
Coefficient of digestibility of the proteins, per cent	81	84
Dietary methionine unabsorbed, per cent	49	47
In vitro enzymic hydrolysis of proteins (a)		
After 1 day, per cent	11	14
After 2 days, per cent	16	25
After 5 days, per cent	22	37
Amino acid composition of the proteins (b)		
Leucine, per cent	7.1	7.7
Lysine, per cent	5.1	5.4
Methionine, per cent	2.2	2.2
Liberation of amino acids (c)		
Leucine, per cent, after 1 day hydrolysis	28	33
Lysine, per cent, after 1 day hydrolysis	45	56
Methionine, per cent, after 1 day hydrolysis	5	10
Leucine, per cent, after 5 days hydrolysis	52	65
Lysine, per cent, after 5 days hydrolysis	77	84
Methionine, per cent, after 5 days hydrolysis	36	73

(a) Based upon liberation of free amino groups, as determined by formol titration.
(b) Analyses on acid hydrolyzed samples, values calculated to 16 per cent nitrogen.
(c) Includes not only free amino acids but also peptides, liberated during the *in vitro* enzymic hydrolysis and not precipitated by 7 per cent trichloroacetic acid. The values listed are percentages of the total.

it was found that the protein in the raw soy had a biological value of 53 per cent as compared with the value of 71 per cent for the heat-processed soy meal.[2] The coefficients of digestibility of the proteins were approximately the same, 81 and 84 per cent, respectively. Calculations based upon the methionine (*1, 10*) intake, the metabolic fecal excretion of the amino acid, and the total fecal methionine excretion indicated that in both cases approximately one-half of the dietary methionine was not absorbed from the gastrointestinal tract. This constitutes a specific case where degree of digestibility of the protein in the gastrointestinal tract as well as the extent of absorption of the limiting amino acid, methionine, are unchanged, but, nevertheless, the biological value (retention of absorbed nitrogen) varies considerably.

The *in vitro* digestibility tests indicated a marked difference in the susceptibility of these proteins to enzymic digestion. This would indicate that during digestion *in vivo* the methionine is released earlier from the heat-processed soy meal than from the raw soy meal. Eventually the same amount of methionine is released and absorbed from either product. However, in the case of the raw meal, absorption occurs so late in the intestinal transit that this amino acid, as well as the incompletely supplemented amino acids, are not efficiently utilized for the synthesis of body protein.

The validity of this interpretation is supported by further experimental findings. The proteins of the raw and processed samples were analyzed for three amino acids, leucine,[3] lysine (*4*), and methionine (*1, 10*). Practically the same values were obtained for both products, in agreement with the values reported in the literature for soy protein (*3*). The deproteinized filtrates obtained in the course of the *in vitro* enzymic hydrolysis of protein, at the end of the first and fifth days, were also subjected to amino acid analysis. The values for leucine, lysine, and methionine in the digests include these amino acids not only in the free state but also in peptide linkage not precipitated by 7 per cent trichloroacetic acid. Hence, the percentage amino acids in the filtrates of the protein digests should be considerably greater than that estimated by formol titration which measures only free amino groups. It may be noted (see Table 2) that methionine was liberated very slowly from raw soy as compared with leucine, while lysine was most rapidly released. In the case of the processed soy the same relationship held, although all amino acids were liberated at somewhat faster rates. When the five-day digests were tested, it was found that most of the lysine and more than half of the leucine had been released. The digest of the heat-processed soy meal contained twice as much methionine as that of the raw meal. These data indicate that methionine is liberated at a slower rate than the leucine or lysine in soy protein and that heat processing increases the rate of liberation of the methionine to a relatively greater extent.

This concept of rate of liberation of methionine supports observations made by others in studies with soy protein. Johnson, Parsons, and Steenbock (*9*) have reported negligible decreases in fecal sulfur and nitrogen when raw soy meal is replaced with a properly cooked product. However, greater retention (decreased urinary excretions) of both these factors was noted when the heat-processed soy meal was fed. These observations are in complete harmony with the hypothesis presented in this report, since they, too, indicate no increase in methionine absorption when

[2] The proteins were included in the diet to the extent of 9 per cent of the ration. Adult male rats were employed for the assays.

[3] Composite of the methods of S. Shankman. *J. biol. Chem.*, 1943, **150**, 305; and J. R. McMahan and E. E. Snell. *J. biol. Chem.*, 1944, **152**, 83.

the heated soy is ingested but, under such circumstances, better utilization of this as well as the other amino acids. More recently, Evans (*6*) reported no correlation between the "digestible" organic sulfur content of soy protein and its nutritional value.

The present studies, conducted with soy protein, have been extended to oats, milk, and other proteins and have yielded similar data indicating that the rate of release of individual amino acids during enzymic digestion can readily account for differences in the biological value of the proteins. However, some precautions must be exercised before rating a sample with maximal digestibility as the most acceptable. For example, excessive heat processing of soybean meal can lead to destruction of some amino acids, so that the biological value becomes the resultant of two factors: (1) the improved digestibility of the protein, leading to greater retention of the absorbed nitrogen, and (2) the imbalance in essential amino acid composition, responsible for a lowering of the biological value of the protein.

On the basis of the present findings it might seem that protein hydrolysates should be superior in biological value to the original intact proteins. In the case of the former, the amino acids would be liberated *in vivo* very rapidly and therefore be available for absorption almost as a group. However, the recent report by Woolley (*13*) indicates that there may be one or more essential peptides which the animal organism is unable to synthesize. It was found that the omission of the factor, strepogenin (presumably a peptide), from a dietary containing a hydrolysate which furnished all of the essential amino acids in satisfactory quantities was responsible for the poor biological response. Thus, in addition to a proper balance of the essential amino acids, it may be necessary to insure certain peptide linkages in the dietary for satisfactory utilization of amino acid nitrogen.

Addendum: The report by Ham and associates (*7*), which appeared while this paper was in press, suggests that the effect of heat on a proteolytic inhibitor in soybean is responsible for the differences in susceptibility of soy protein to enzymic digestion. However, the impairment in utilization of the protein derived from raw or improperly heat-processed meals must be attributed to an inhibition of the *rate* of methionine liberation for mutual amino acid supplementation and not to a reduced *degree* of methionine availability as postulated by Ham and collaborators.

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A "Frenching" Response of Tobacco Seedlings to Isoleucine

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Growth of tobacco seedlings in aseptic cultures under constant light and temperature was found to be greatly modified by the presence of isoleucine in the culture medium. The abnormalities in gross morphology resulting from the addition of this amino acid consisted in loss of dominance of the apical bud, production of numerous quite narrow leaves, and mottled chloroses. The shortened stem and excessive development of axillary buds gave a "witch's broom" type of growth. The abnormalities enumerated are characteristic of a tobacco disease called frenching, the cause of which is unknown.

Slight variations from the symptoms of frenching in the field were observable under the extreme environmental conditions employed. These abnormal conditions were very high moisture and relative humidity, low light intensity, and the use of agar instead of soil. The mottling of the leaves was not of the reticular pattern typical of frenching, nor did the narrow or strap-leaves assume the upright position to the same degree. There was little indication of wavy margins or ruffling of strap-leaves.

These abnormalities occurred regularly in every experiment with several samples of this amino acid. None of the other amino acids, sugars, vitamins, and peptones (a total of 60) tested brought about this reaction. The severity of abnormal symptoms varied with the concentration of isoleucine employed. Levels of 50 to 200 p.p.m. proved adequate under these conditions. Complete details of the experimental work will be reported later.