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Is pH Drop a Valid Measure of Extent of Protein Hydrolysis?

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Utilizing nine foods as substrates, protein digestibility as determined by the two-step, pH-drop procedure of Satterlee et al. was compared to the extent of hydrolysis at the end of the incubation period. The extent to which the substrate protein was hydrolyzed was found to be much less than the calculated digestibility, as expected. However, the digestibility and extent of hydrolysis did not correlate with one another in any reasonable way. It is concluded that, although pH drop can be used to follow the course of hydrolysis of a single substrate, and in spite of a demonstrated correlation between pH drop and in vivo protein digestibility, pH drop alone cannot serve as a measure of extent of protein hydrolysis for the comparison of different substrates.

Since evaluation of the protein digestibility of food products by in vivo methods is very slow and costly, a quick, reliable in vitro procedure to replace them has been sought. Satterlee and co-workers (Satterlee and Kendrick, 1979; Satterlee et al., 1979, 1982) have proposed a procedure in which protein digestibility is calculated from the drop in pH obtained after in vitro digestion of the substrate, initially at pH 8.00, with pancreatic, intestinal, and bacterial enzymes. The correlation between their in vitro results and those obtained in vivo is impressive.

In our laboratory we observed that the digestibility of various substrates as determined by the procedure of Satterlee et al. seemed to bear little or no relation to the extent of protein hydrolysis after digestion of the same substrates with pepsin, Pronase, and kidney peptidase (Mozersky and Panettieri, 1983). There are two possible explanations of this: (1) The digestion procedure used by Satterlee et al. is very different from our own. (2) The pH drop obtained on hydrolysis of a protein is dependent on factors other than extent of hydrolysis and cannot be used as a measure of the extent of protein hydrolysis. The latter possibility is examined in the work presented here.

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MATERIALS AND METHODS

Materials. The trypsin, chymotrypsin, and peptidase used were the products specified by Hsu et al. (1977). The bacterial protease was Sigma Chemical Co. Type XIV (Pronase E) from *Streptomyces griseus*, the product specified by Satterlee et al. (1982).

Substrates are listed in Table I with their proximate compositions. All of the substrates are solids with a particle size sufficiently small to pass an 80-mesh screen, with the exception of the brain preparation, which was ground to pass a 2-mm screen.

Assays for Total Nitrogen and for Amino and Amide Group Content. Nitrogen content was determined by Kjeldahl digestion followed by assay with ninhydrin (Jacobs, 1962). Twenty-milligram samples of the substrates were digested according to the procedure of Willits

Table I. Proximate Composition of Substrates (Percent)[a]

substrate	moisture[d]	ash[d]	fiber[d]	fat[d]	nitrogen[e]	protein[f]
1, blood plasma	8.87	9.83		0.13	12.02	75.1
2, casein, ANRC[b]	4.36	1.06	0.42	0.00	13.76	86.0
3, peanut flour	4.90	4.90	4.43	0.06	9.35	58.4
4, wheat flour	8.00	0.50	0.85	1.12	1.70	10.6
5, heart	4.07	4.20		10.28	11.80	73.8
6, brain	4.03	6.12		39.86	7.70	48.1
7, Enrpro-50	6.66	10.09		0.07	8.01	50.1
8, lactalbumin	3.99	1.03	0.48	2.19	12.29	76.8
9, caseinate, FCC[c]					13.70	85.6

[a] On an as-is basis. [b] ANRC = Animal Nutrition Research Council. [c] FCC = first cycle casein (sodium caseinate), prepared by Pepper according to his procedure (Pepper, 1972). It contains a trace of Ca, no α-lactalbumin, and no β-lactoglobulin. [d] Data of Hackler et al. (1983), received as a personal communication from M. L. Happich. These analyses were done approximately 4 years before the work reported here and are presented for general information. Substantial changes in the moisture content of some of the substrates undoubtedly occurred during that time, with corresponding changes in the contents of the other components. [e] Determined by the authors. [f] Defined as 6.25 × nitrogen.

and Ogg (1950). The digests were diluted with water to 50 mL, and insoluble material was allowed to sediment under gravity. One hundred microliter aliquots of the supernatants were assayed by the manual ninhydrin method of Moore (1968).

As an estimate of peptide bond content, the amino plus amide group content of the substrates was determined with ninhydrin after digestion of 5-mg samples with 5.7 N HCl in evacuated tubes for 24 h at 110–115 °C (Matoba et al., 1982). After removal of HCl, the residues were dissolved in 5 mL of 2 M lithium acetate buffer, pH 5.2, and filtered through 0.45-μm Millipore filters. One hundred microliter aliquots of the filtrates were assayed in the same manner as the diluted Kjeldahl digests.

Assay for Digestibility. The digestibility of each substrate was determined, in duplicate or triplicate, by the improved, two-step, pH-drop procedure of Satterlee and co-workers (Satterlee and Kendrick, 1979; Satterlee et al., 1979, 1982). To facilitate later discussion, this procedure can be summarized as follows: the substrate, at an initial pH of 8.00, is digested sequentially with (a) a mixture of mammalian proteases and (b) a bacterial protease preparation. Each incubation period is 10 min. Incubation a is at 37 °C. Incubation b is at 55 °C for 9 min followed by 37 °C for 1 min. At the end of the second incubation, i.e., at total incubation time t = 20 min, the pH, $(pH)_{20}$, is measured. Protein digestibility is calculated with the equation

$$D = 234.84 - 22.56(pH)_{20} \quad (1)$$

Incubations were carried out with continuous stirring in a semi micro jacketed flask, Radiometer type V 533. Two constant-temperature baths provided circulating water at the required temperatures, 37 and 55 °C. A two-position selector valve, designed for the purpose, permitted connecting either source to the jacket of the incubation vessel. Switching from one position to the other took about 1 s. pH was measured with Radiometer electrodes G 2040C and K 4040 attached to a Radiometer RTS 822 autotitration system; the pH meter was a Model pHM 84. The autotitration system permitted the initial adjustment of the pH to 8.00 to be made automatically.

Assay for Extent of Hydrolysis. To provide samples for measurement of the extent of protein hydrolysis, an equal volume, i.e., 12 mL, of 10% trichloroacetic acid (TCA) was added to each digest immediately (within 10 s) after the terminal pH reading at incubation time t = 20 min. The acidified digest was centrifuged twice for 25 min at 10 °C and 35000g to remove precipitated proteins. For the controls, TCA was added to the substrate solution or suspension prior to addition of the enzymes. After dilution of each deproteinized sample with 3 volumes of water, duplicate or triplicate 100-μL aliquots were assayed for amino group ($+NH_3$) content with the manual ninhydrin method described by Moore (1968). Omission of the deproteinization step prior to assay with ninhydrin gave erratic results, probably because of hydrolysis of larger peptides during the assay procedure (Hirs, 1967). Glycine (12 mM) was used as standard, being treated as were the deproteinized digests and controls. Absorbance was measured at 570 nm with a Gilford Model 2000 multiple absorbance recording system. Readings were linear with amino group concentration to an absorbance of 2.8.

The extent, E, of enzymatic digestion (protein hydrolysis) was calculated as follows:

$$F = \frac{100(N/m)}{(N/m)_{Kj}} \quad (2)$$

$$E = F - F_c \quad (3)$$

where N = the amount (μmol) of amino groups plus NH_3 in the digest or control, and m = the mass (mg) of substrate subjected to digestion. In the expression for F, the numerator refers to the enzymatic digest (or control). The denominator refers to a Kjeldahl digest of the same substrate, as indicated by the subscript Kj. The subscript "c" designates "control". E therefore expresses the number of moles of amino group released enzymatically per 100 mol of Kjeldahl nitrogen.

An alternative evaluation of the extent of enzymatic digestion (protein hydrolysis) was calculated as follows:

$$F' = \frac{100(N/m)}{(N/m)_{HCl}} \quad (4)$$

$$E' = F' - F_c' \quad (5)$$

The denominator in the expression for F' refers to the HCl digest of the substrate. Otherwise the symbols have the same meanings as in eq 2 and 3. E' therefore expresses the number of moles of amino groups plus NH_3 released by enzymatic digestion per 100 moles of amide plus amino groups in the substrate.

RESULTS AND DISCUSSION

Relative Values of *D* and *E*. The expression (eq 1) relating protein digestibility, D, to terminal pH, $(pH)_{20}$, is an empirical one. $(pH)_{20}$ was measured in Satterlee's laboratory for a wide variety of substrates of known in vivo (apparent nitrogen) digestibility, and eq 1 is the best linear fit for this set of paired values. Thus, D provides no information as to the extent to which the substrate protein has been hydrolyzed in vitro. It was, therefore, of interest to experimentally compare the digestibility, D, with the extent of protein hydrolysis in the Satterlee digest at total incubation time t = 20 min.

Table II. Ratio of Protein Digestibility, D, to the Extent of Digestion

substrate	D/E	D/E'
1, blood plasma	2.66	1.96
2, casein, ANRC	2.27	1.76
3, peanut flour	3.16	2.43
4, wheat flour	3.16	2.74
5, heart	2.26	1.71
6, brain	2.92	1.81
7, enrpro	2.48	1.92
8, lactalbumin	2.60	2.22
9, caseinate, FCC	2.33	1.64

Apparent protein (or nitrogen) digestibility is defined (Hopkins, 1981) as

$$\frac{100(\text{nitrogen intake} - \text{fecal nitrogen})}{\text{nitrogen intake}}$$

Since D is a prediction of this quantity, the enzymatic digestion of various substrates is run with a constant amount of substrate nitrogen, viz., 10 mg (Satterlee and Kendrick, 1979). The extent of digestion, E, to be compared to D was therefore defined on the basis of the total nitrogen content of the substrate (eq 2 and 3).

The comparison of protein digestibility, D, as measured in our laboratory by the method of Satterlee et al., to the extent of protein hydrolysis, E, measured as described under Materials and Methods, is shown in Table II. D is thus found to be from 2.3 to 3.2 times as large as E. The prediction of in vivo protein digestibility is thus based on an in vitro digestion which is less than half as extensive as that achieved in vivo. Such a prediction is inherently risky, and it is, perhaps, surprising that, at least for the substrates investigated by Satterlee and Kendrick (1979), D correlates with in vivo digestibility as well as it does.

It may be objected that E is artificially low, and D/E therefore artificially high, because, after enzymatic digestion, only the free amino group plus NH_3 content of the TCA-soluble fraction was measured, not its total nitrogen content. We can calculate the extent of hydrolysis in terms of the total nitrogen content of the TCA-soluble fraction relative to that of the untreated substrate by multiplying F (eq 2) by $(N/m)_{Kj}/(N/m)_{HCl}$. (For definitions of terms see the text relevant to eq 2 and 4.) This yields

$$F' = \frac{(N/m)_{Kj}}{(N/m)_{HCl}}F$$

$$F' = \frac{100(N/m)}{(N/m)_{HCl}}$$

which is eq 4. The extent of hydrolysis, E' (eq 5), is then in the desired terms. This treatment, of course, assumes that the ratio $(N/m)_{Kj}/(N/m)_{HCl}$ is, for a given substrate, the same for the TCA-soluble fraction of the enzymatically digested substrate as it is for the insoluble fraction. E' has as its basis the amino group plus NH_3 content of the substrate after hydrolysis with HCl and can be regarded as an estimate of the percentage of peptide bonds in the substrate protein that have been hydrolyzed enzymatically. The ratio D/E', shown in the last column of Table II, varies from 1.7 to 2.7. Thus, even by this measure, D is substantially greater than the extent of hydrolysis.

Correlation between D and E. The fact that D/E (or D/E') $\gg 1$ does not mean that D is necessarily incorrect. It is not even necessary that the ratio be constant for various substrates. It is conceivable that digestibility might be a linear function of $(pH)_{20}$, as required by eq 1, even if it were not directly proportional to the extent of hydrolysis, E or E'. However, the basic concept historically

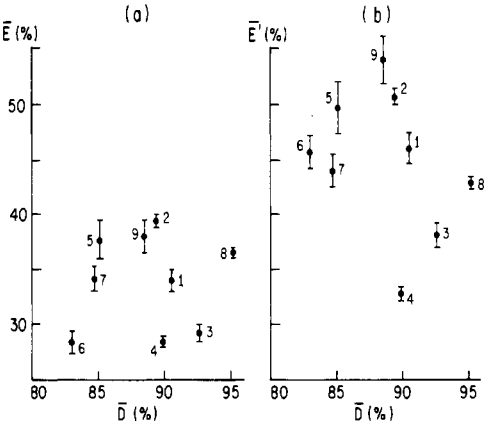
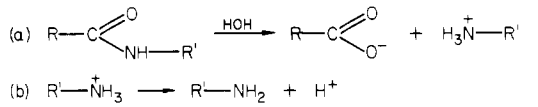


Figure 1. Extent of digestion as a function of protein digestibility. (a) Extent of digestion, E, was calculated on the basis of the total nitrogen content of the substrate; see eq 2 and 3. (b) Extent of digestion, E', was calculated on the basis of amino groups plus NH_3 released on digestion with HCl; see eq 4 and 5. The numbers adjacent to the data points identify the substrates; see Table I, column 1. The vertical bars are standard deviations.

underlying in vitro procedures such as that of Satterlee et al. is the assumption that digestion of proteins in vivo by proteases in the lumen and wall of the digestive tract can be simulated by the action of proteases in vitro, the extents of digestion in vivo and in vitro being at least positively correlated. Parts a and b of Figure 1 show plots of E vs. D and E' vs. D for the nine substrates used in this investigation. It is quite apparent that, in fact, no reasonable correlation exists between D and the extent of hydrolysis. This visual impression is confirmed by statistical analysis of the data. In order to take into account variability in D as well as variability in E and E', all individual data points (D, E) and (D, E') were included in the analysis rather than the average values shown in Figure 1. The correlation coefficients for E vs. D and E' vs. D are 0.13 and −0.29, respectively. The former is not distinguishable from zero at any reasonable level of significance. The latter is distinguishable from zero at the 1.7% level of significance but, being negative, is not compatible with any reasonable relationship between extent of hydrolysis and digestibility.

The lack of correlation between digestibility, as calculated from the terminal pH, and extent of hydrolysis is attributable to the fact that the number of peptide bonds ruptured is not the only determinant of the drop in pH. The latter is dependent on the number and pK values (Chance and Nishimura, 1967) of (1) amino groups released, (2) initially buried buffering side chains exposed on hydrolysis, and (3) buffering side chains present in exposed form in the unhydrolyzed protein. As an illustration of the influence of the pK values of (1), consider two hypothetical proteins, both initially at pH 8, in each of which 50% of the peptide bonds have been hydrolyzed. If the amino groups liberated in the one protein all have a pK_a of 7.5 while all those in the other protein have a pK_a of 8.5, the pH in the former case would be expected to drop substantially further than in the latter case. Otherwise stated, peptide bond hydrolysis can be treated as consisting of two steps



In the terminal pH range observed in practice, viz., approximately 6–7, virtually all carboxyl groups are dissociated and can be neglected. The difference in the pH

values of the two hypothetical protein digests is due to the difference in pK_a values for step b. Differences in the number and pK values of (2) and (3) can likewise lead to different pH drops in two proteins hydrolyzed to the same extent.

The lack of correlation between the digestibility, D, and the extent of protein hydrolysis, E or E', brings into question the validity of D as a predictive measure of in vivo digestibility. This lack of correlation is, in principle, ascribable to the methodology involved in determining E and E'. However, ninhydrin analysis of TCA-soluble peptides and amino acids is an accepted, indeed recommended, procedure for evaluating the extent of protein digestion (Reimerdes and Klostermeyer, 1976). For the reasons discussed above, it appears reasonable, in the absence of evidence to the contrary, to attribute the lack of correlation between D and E (or E') to a weakness in the former rather than the latter.

Equation for Digestibility. To emphasize the relationship of digestibility to the drop in pH during the 20-min incubation period, eq 1 can be rewritten as

$$D = 54.36 + 22.56[8.00 - (pH)_{20}]$$

where the term is brackets is the drop in pH. It is thus apparent that if the terminal pH were identical with the initial pH, the calculated digestibility would be not 0, as one would expect, but 54.36%. The terminal pH for which a digestibility of 0 would be calculated is directly apparent by rewriting the equation in the form

$$D = 22.56[10.41 - (pH)_{20}]$$

from which $D = 0$ at $(pH)_{20} = 10.41$. Thus, in the absence of enzymatic digestion, the pH would be expected to rise from 8.00 to 10.41. These peculiar features of D can be explained by assuming that, during digestion of the substrates investigated by Satterlee et al., in addition to hydrolysis of peptide bonds, which lowers the pH from the initial value of 8, there was also exposure of unprotonated basic groups, which tends to raise the pH. However, it is questionable whether such buried groups are so widespread as to become manifest in an equation (eq 1) based on measurements made with a wide variety of protein-containing substrates.

CONCLUSION

The correlation of the pH drop observed on digestion of a protein-containing substrate with its protein digestibility does not appear to result from a simple and well-defined relationship between each of these quantities and the extent of protein hydrolysis, as sometimes assumed. The limits within which the pH-drop method for predicting protein digestibility is valid are, therefore, not known. Consequently, caution should be exercised in applying the pH-drop method to substrates whose in vivo digestibility has never been measured.

Registry No. Proteinase, 9001-92-7.

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