CXIV. THE ACTION OF PEPSIN ON GELATIN.

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THERE can be little doubt that the chief chemical change which accompanies the digestion of protein by the enzymes of the pancreas and intestine and by the endocellular proteases is the hydrolysis of acid amide (*i.e.* peptide) linkages. This conclusion rests securely upon the fact that carboxyl and amino-groups are liberated in large numbers and in equivalent amounts. The magnitude of these chemical effects, moreover, has been found to run more or less parallel with the extent of the "physical digestion" of the protein which is betrayed in changes in solubility, diffusibility, viscosity, etc. In these respects, however, the behaviour of pepsin is peculiar. Pepsin is able to accomplish quite extensive degradation of protein without effecting any significant increase in titratable groups. This situation has led, from time to time, to the suggestion that the specific character of peptic digestion lies in the disintegration of the protein molecule into smaller fragments which were united, in the original molecule, by forces other than those of primary valency. Such a view is difficult to prove or to deny. It may be suggested, however, that it is a view whose utility becomes questionable once it has been definitely established that protracted digestion of protein is accompanied by regular and orderly increases in acidic and basic groups.

That such increases do occur has been demonstrated repeatedly, but the evidence respecting their orderliness has been somewhat conflicting. In particular, the report has frequently been made that the carboxyl groups liberated are in considerable excess of the amino-groups simultaneously set free. This has led to the impression that there may occur in the protein molecule linkages in which —COOH is united to some other group than primary —NH_2 .

Within recent years there has been a revival of interest in the question owing to general recognition of the fact that its solution has an important bearing upon the general problem of protein structure. In 1926 two papers [Steudel, Ellinghaus and Gottschalk, 1926] appeared in which were reported studies of the peptic digestion of fibrin, edestin, vitellin, caseinogen, serum-albumin, serum-globulin, gluten and gelatin. In every case the authors found that the ratio of liberated groups, $\frac{\text{COOH}}{\text{NH}_2}$, exceeded unity. In the early stages of digestion it sometimes attained values as great as 7. Waldschmidt-Leitz and Simons [1926] also observed an excessive liberation of —COOH in the

cases of gelatin, gliadin and zein. In the digestion of caseinogen, egg-albumin and ricinus-globulin, on the other hand, the hydrolysis ratio approximated to unity. Meanwhile Steudel and Ellinghaus [1927] confirmed their original observations in a further paper. Felix and his collaborators [Felix and Harteneck, 1927; Felix and Muller, 1927] reported that the peptic digestion of thymus histone was accompanied by an equivalent increase in acidic and basic groups. Discrepancies between the increases in total acid-binding power and in free —NH_2 groups led them, however, to suggest the participation of the guanidine group of arginine in linkages with —COOH . This suggestion received no support from Waldschmidt-Leitz and Kunstner [1927], who arrived at the conclusion that the only important chemical changes which accompany peptic digestion are the hydrolysis of peptide linkages. This view was further supported by the observations of Felix and Buchner [1927] on haemoglobin, of Weber and Gesenius [1927] on caseinogen and of Sørensen and Katschioni-Walther [1928] on caseinogen, gliadin and gelatin. These last authors discuss in detail a number of errors which attend the application to protein solutions of the usual methods of determining —COOH and —NH_2 groups. They conclude that it has been an inadequate appreciation of these errors which has been responsible for the various observations in which the numbers of equivalents of liberated —COOH and —NH_2 have been found to be unequal.

The balance of evidence clearly favours the view that pepsin, like other proteolytic enzymes, is a true peptidase. It is, however, disturbing when different observers, using similar methods of analysis, arrive at such conflicting results. There remains a feeling that the methods may not be well adapted to the purposes for which they have been employed.

The determination of —COOH has always been made by titration of the digests with alkali in the presence of alcohol, formaldehyde, acetone or mixtures of these solvents. For the determination of —NH_2 groups, use has been made of some form of titration with acid such as that of Linderstrøm-Lang [1928], of Folin's colorimetric method or of the nitrous acid method of Van Slyke. The uncertainty of the two latter methods when applied to proteins is well known. It must be conceded, also, that the various titrimetric methods are equally empirical since they depend upon the titration of acidic and basic groups to arbitrary end-points in mixed solvents in which the controlling acid-base equilibria have not been adequately defined. It is to be observed, however, that their use rests upon the assumptions that such equilibria do exist and that the reaction of proteins and their digestion products with acids and bases are stoichiometric under the empirically chosen conditions. In view, therefore, of the success which has attended the quantitative treatment of proteins as ampholytes, it is surprising that no attempt appears to have been made to establish the characteristics of peptic digestion by direct electrometric titration of the aqueous digests. In the present paper such an attempt will be described.

EXPERIMENTAL.

Gelatin was chosen as substrate since it offered an unusual combination of advantages. It is readily and extensively digested by pepsin. It is soluble over the whole experimental p_H range and its digestion is not complicated by the separation of insoluble plastein material. The general form of its titration curve is well known and the interpretation of changes which are found to occur upon digestion is not confused by the presence of such ionising groups as the phenolic hydroxyl and thiol groups or by large numbers of amide groups. It has, moreover, been in the case of gelatin that many of the conflicting results, to which reference has been made, have been obtained.

Isoelectric gelatin was prepared by the method of Loeb from three different commercial samples. In the various experiments, also, three different commercial samples of pepsin were employed. The composition of the initial digest mixtures was varied with respect to the concentration of gelatin (gelatin-nitrogen 0.30–0.84 %), the concentration of pepsin (pepsin-nitrogen 0.003–0.03 %) and the initial p_H (1.6–3.1). The ratio of pepsin-nitrogen to gelatin-nitrogen did not, in any experiment, exceed 0.09 and was reduced in some below 0.01. In the latter cases, the increases in acid-binding capacity which accompanied digestion were in considerable excess of the total nitrogen of the pepsin. Consequently there was no possibility that changes in the enzyme material were responsible for the effects observed. The digestion mixtures were preserved with toluene and incubated at 38°. At intervals up to 14 days samples were removed for analysis. All observations were controlled by determinations on similar samples from identical mixtures containing inactivated enzyme. The latter was prepared either by heating the solution of enzyme or by rendering it alkaline to p_H 9 for 15 minutes and then adding an equivalent of acid. The latter method had the advantage of avoiding the introduction of coagulated matter into the control mixtures. Some or all of the following determinations were made on each analytical sample: the hydrogen electrode titration curve, the formaldehyde titration (Sørensen), amino-nitrogen (Van Slyke), viscosity, time of gelation at 0° at the isoelectric point, and nitrogen soluble in four volumes of alcohol at the isoelectric point.

The last three determinations were intended to control the rate of change in the physical properties of the hydrolysate. In this way it was hoped to provide a commentary upon the more intimate chemical changes revealed by analysis.

Hydrogen electrode titration.

Platinised or palladinised platinum electrodes were employed. The reference electrode was a saturated calomel half-cell connected to the unknown solution by a saturated potassium chloride bridge. The calomel cell was standardised against 0.1 *M* hydrochloric acid, the p_H of the latter being taken to be 1.08 between 25 and 38°. Two methods of titration were followed.

Method I. To small aliquot volumes of the digest were added varying amounts of standard sodium hydroxide and the mixture was diluted to a standard volume. The hydrogen electrode potentials of these mixtures were determined in an electrode vessel of the type designed by Clark. New electrodes were brought into service at the first indication of sluggish reaction or lack of reproducibility.

Method II. The second method was more economical of time and material and appeared to be equally accurate. 20 cc. of digest were placed in a small titrating bottle containing a Bunge electrode. Such a volume of sodium hydroxide was added as would bring the mixture to about p_H 11 and the air space was displaced with purified hydrogen. The alkali served to destroy the enzyme and so insure that no further digestion occurred during the progress of a titration. The mixture was then titrated with 0.1 *M* hydrochloric acid, potentials being determined after each addition. A new electrode was substituted whenever there was reason to question the activity of that in use. In general, one electrode survived a complete titration. Initial equilibrium required about 30 minutes but adjustments to additions of acid were usually complete within a few minutes.

In the second method of titration it was anticipated that loss of fluid through frothing would occur. As a matter of fact frothing never became uncontrollable. Since, however, a modified electrode was devised to meet the contingency its description may be of interest. The modification consisted in providing a by-pass to the hydrogen supply to the electrode. This may conveniently take the form of the glass portion of another Bunge electrode immersed in water to a depth just less than that to which the hydrogen electrode is immersed in the experimental solution. When the gas supply is turned on the levels of the fluids in the two electrode tubes pulsate together but hydrogen escapes only from the by-pass. Excessive agitation of the protein solution is, thereby, avoided. It is important that the gas train be constructed of metal or other tubing not permeable to gases and that it be liberally swept out with hydrogen at the beginning of the experiment. A few bubbles of hydrogen, also, should be allowed to escape into the solution at short intervals, this being effected by momentarily closing the by-pass. This precaution is taken to meet the tendency of traces of heavier gases to be displaced downwards to the electrode surface. The modified electrode worked well in practice.

Titration curves.

The "corrected equivalents of base" (b') in combination with 1 g. of gelatin at the various determined p_H values have been calculated from the relation

$$b' = b - a + h - oh,$$

where a and b are the respective concentrations of added hydrochloric acid and sodium hydroxide and h and oh are the concentrations of hydrogen and

hydroxyl ions respectively. The last two were calculated from the observed hydrogen ion activities with the aid of the relations

$$p_h = p_H - \log \gamma \text{ and } p_H + p_{OH} = p_{kw}.$$

The activity coefficient of the ion concerned, γ , was calculated from the simplified equation of Debye and Huckel [Simms, 1926]

$$\log \gamma = 0.3 \sqrt{\mu}.$$

In computing μ , the ionic strength of the solution, the contribution of the protein ions was arbitrarily ignored.

For economy of presentation a graphic method of analysis of the titration curves may be adopted. Only one experiment will be quoted in detail. Fig. 1

Fig. 1. Titration curves of peptic digests of gelatin. (a) Curve of 24 hours' control sample. (b) Curve of 24 hours' digest sample. (c) Curve of 48 hours' digest sample. (d) Curve of 7 days' digest sample.

presents the titration curves of a digestion mixture at various stages of digestion. The equivalents of acid ($-b'$) and of base ($+b'$) bound to 1 g. of gelatin are plotted against the experimental values of p_H . From these curves have been graphically derived the values of b' corresponding to equal increments of p_H (Table I). The large number of experimental points reduces the danger of such a graphic analysis significantly distorting the data.

Since pepsin does not carry the digestion of protein beyond the stage of complex polypeptides we are entitled to assume that, in the hydrolytic products, the original ionising groups are still separated from one another and from the extra liberated groups by relatively great molecular distances.

Table I.

Digest mixture		0.32 % gelatin-nitrogen 0.029 % pepsin-nitrogen		} p_H 2.4.			
		$b' \times 10^6$ per g. gelatin					
Total				Increment			
p_H	Control 24 hr	Digest			Digest		
		24 hr	48 hr	7 day	24 hr	48 hr	7 day
2.0	- 117.0	- 145.5	- 153.5	- 173.0	- 28.5	- 36.5	- 56.0
2.4	- 109.0	- 137.0	- 146.0	- 164.0	- 28.0	- 37.0	- 55.0
2.8	- 99.0	- 123.0	- 131.5	- 147.0	- 24.0	- 32.5	- 48.0
3.2	- 85.5	- 103.5	- 109.5	- 123.0	- 18.0	- 24.0	- 37.5
3.6	- 65.5	- 80.0	- 83.0	- 94.0	- 14.5	- 17.5	- 28.5
4.0	- 45.5	- 54.0	- 56.0	- 64.5	- 8.5	- 10.5	- 19.0
4.4	- 25.0	- 30.0	- 31.0	- 37.5	- 5.0	- 6.0	- 12.5
4.8	- 7.5	- 8.5	- 10.0	- 14.5	- 1.0	- 2.5	- 7.0
5.2	+ 5.0	+ 6.5	+ 4.5	+ 1.5	+ 1.5	- 0.5	- 3.5
5.6	14.0	16.0	15.0	10.5	+ 2.0	+ 1.0	- 3.5
6.0	19.5	23.0	22.5	18.5	3.5	3.0	- 1.0
6.4	24.0	29.5	29.0	26.0	5.5	5.0	+ 2.0
6.8	28.0	37.0	38.0	37.0	9.0	10.0	9.0
7.2	32.0	47.0	48.5	49.5	15.0	16.5	17.5
7.6	36.5	57.0	59.5	63.5	20.5	23.0	27.0
8.0	41.0	65.5	70.0	76.0	24.5	29.0	35.0
8.4	45.5	72.0	77.5	87.0	26.5	32.0	41.5
8.8	50.0	78.5	85.0	97.5	28.5	35.0	47.5
9.2	56.0	86.0	92.0	106.5	30.0	36.0	50.5
9.6	63.5	94.5	100.5	115.5	31.0	37.0	52.0
10.0	72.0	104.0	110.0	126.0	32.0	38.0	54.0
10.4	80.5	112.5	119.0	135.0	32.0	38.5	54.5
10.8	84.0	116.5	122.5	138.5	32.5	38.5	54.5

Consequently, their "strengths" should not be profoundly modified by the appearance of these new groups. Provided, therefore, there that has been no simultaneous condensation of free groups, we should be able to arrive at an approximation not only to the amounts but also to the "strengths" of the liberated groups by subtracting from one another the curves of the protein before and after digestion. This has been done in Table I. The resultant figures have, in one case (7 days digest), been charted as discrete points in Fig. 2. The continuous curve which has been superimposed upon these points is the theoretical curve for an ampholyte having $p_{K'}$ values of 3.5 and 7.5 and a concentration equal to the increment in equivalents of base bound by the protein at p_H 10. These $p_{K'}$ values proved to be the best for all titrations conducted at 38°. In other experiments, in which titrations were carried out at 25°, it was found that the best fit was obtained by assuming constants of 3.5 and 7.9. This shift of $p_{K'}$ with temperature may be taken to be interesting confirmation of the conclusion that the liberated groups which ionise in this region are basic groups since the two values which have been quoted both correspond to a p_{K_b} value of 6.0.

The data which have been presented seem to establish quite definitely that the ratio of the increments in acid- and in base-binding capacities of gelatin closely approximates to unity at each stage in the digestion. Moreover, it is suggested that the mean dissociation constants of the acidic and basic

groups set free are closely represented by $p_{K_1'} = 3.5$ and $p_{K_2'} = 7.9$ (at 25°) respectively. Since all experiments led to the same unequivocal result, it may be taken to be independent of the composition of the digest within the limits stated at the outset. It remains to compare the mean dissociation

Fig. 2. "Titration curve" of groups liberated in peptic digestion. The continuous curve is the theoretical curve for an ampholyte having $p_{K'}$ values of 3.5 and of 7.5 (at 38°). The corresponding values at 25° were found to be 3.5 and 7.9.

constants which have been derived with those of the $-\text{COOH}$ and $-\text{NH}_2$ groups of peptides of increasing complexity to arrive at the reasonable inference that the main chemical effect of pepsin is the hydrolysis of peptide unions between $\alpha\text{-COOH}$ and $\alpha\text{-NH}_2$ groups of amino-acids (Table II).

Table II. *Dissociation constants of peptides* (from Kirk and Schmidt [1929]).

	p_{K_1}	p_{K_2}
Glycine	2.37	9.72
Alanine	2.35	9.72
Glycylglycine	3.12	8.07
Glycylalanine	3.15	7.9
Glycylglycylglycine	3.26	7.9
Glycylalanylalanylglycine	3.30	7.9
Groups set free by pepsin (cf. text)	3.5	7.9

Apart from this, the major effect of pepsin, two minor effects are suggested by our results. One of these is the progressive displacement of the curve in the isoelectric region as digestion proceeds (Fig. 1). The other is the indication of a small increase in base-binding capacity in the zone p_H 9–10 (Fig. 2). These effects were constantly observed and were absent from the curves of control mixtures. Their magnitude—less than 5×10^{-5} equivalents per g. gelatin even after 7 days—is, however, scarcely greater than the cumulative errors which are involved in our treatment of the data. If we accept 61,000 as the molecular weight of gelatin [Kunitz, 1926–7] it may be calculated that such an increase in b' corresponds to the formation of only two new ionising

groups per molecule. That is to say, our argument does not claim to be able to demonstrate an effect which is restricted to the hydrolysis of only a single protein linkage. At the same time, we may say that the two effects which have been referred to must, if they are real, be quite restricted in their incidence upon the protein molecule.

In conclusion, it should be pointed out that we have chosen to compare the titration curves of all digest mixtures with the initial curve for the control mixture rather than with that of a control of the same age. This has been done merely to permit economy in the reproduction of data. It is justified by the fact that the small changes which do occur in the controls indicate that the small amount of hydrolysis occurring under the influence of the acid present liberates groups of a character similar to those set free in much greater amount by the pepsin.

The relation between the chemical and physical changes in peptic digestion.

Comment was made, in the introduction, on the remarkable physical changes which are found to accompany relatively small increases in titratable groups. This may suitably be illustrated by an experiment in which only a low concentration of pepsin was employed. The viscosity of the mixture fell to one-half its original value in only 90 minutes. Moreover, the time of gelation increased rapidly during the first 4 hours. At the end of 6 hours the digest failed to form a gel at the isoelectric point at 0°. We may, therefore, conclude that unchanged gelatin rapidly disappeared. Within the same period the amount of nitrogen soluble in 80 % alcohol at the isoelectric point (in water) increased rapidly. Only after much longer periods was it possible to demonstrate important increases in titratable groups. The pertinent data are assembled in Table III.

Table III.

Digestion mixture		0.35 % gelatin-nitrogen 0.003 % pepsin-nitrogen		} p_H 1.9.		
Equiv. $\times 10^5$ per g. gelatin						
Digestion time (hours)	Alcohol- soluble nitrogen	Acid bound		Alc. sol. N NH_2 set free	NH_2 groups set free per mol.	Hydro- lysis %
		Total	Set free			
Zero	10	77	—	—	—	—
2	52	—	—	—	—	—
4.5	75	—	—	—	—	—
9	109	84	7	14	4.3	0.7
22	176	88	11	15	6.8	1.1
70	304	96	19	15	11.7	1.9
168	361	102	25	14	15.4	2.4
356	420	107	30	14	18.5	3.0

The last column gives a figure for the percentage of the total hydrolysis which had been accomplished. This is the percentage of the peptide nitrogen of gelatin which is represented by the NH_2 -nitrogen set free. The peptide nitrogen was calculated from the total nitrogen and from Dakin's figures [1920] for the composition of gelatin. The assumption has been made—a necessary

one on the basis of the polypeptide theory of protein structure—that proline and hydroxyproline can form peptide-like unions on their nitrogen atoms. The sixth column gives the number of peptide linkages split per molecule on the assumption that gelatin has a molecular weight of 61,000.

An interesting relation is disclosed by column 5. It will be seen that each peptide linkage hydrolysed rendered soluble in 80 % alcohol 14–15 atoms of nitrogen. A ratio of similar magnitude was found in all experiments in which it was sought provided hydrolysis had not proceeded beyond about 7 % of complete digestion. What significance may be attached to this relation is uncertain in view of the empirical nature of fractionations effected by precipitation with alcohol. It does, however, suggest that the fragments resulting from the early peptic digestion of gelatin are of large mean molecular weight.

We hope to be able to continue the investigation of some of the questions which have been raised.

SUMMARY.

1. The changes in acid- and in base-binding capacities which accompany the digestion of gelatin by pepsin have been determined with the aid of hydrogen electrode titration curves. The results indicate that the main chemical effect of pepsin is the hydrolysis of peptide linkages constituted between the α -amino- and α -carboxyl-groups of amino-acids.

2. Some observations are added correlating the chemical changes with certain changes in the physical behaviour of the protein.

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