THE ENZYMATIC SYNTHESIS OF PROTEIN. III.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION ON PEPTIC SYNTHESIS.

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In two previous communications the authors have described a synthesis of protein by pepsin in a concentrated peptic hydrolysate of albumin (1); and the effect of temperature on this synthesis (2). The justification for describing the synthetic product as protein is discussed in our previous paper (2). In these communications the optimum hydrogen ion concentration was stated to be pH 4.0; but the influence of the hydrogen ion concentration was otherwise not discussed. The importance of the degree of acidity was realized early by Sawjalow (3), who did not, however, define it precisely, and by Henriques and Gjaldbäk (4), who gave the optimum pH as 1.5. This hydrogen ion concentration in our experience, despite the existence of all other optimum conditions, allows only very small amounts of synthesis. Either Henriques and Gjaldbäk were in error, or the occurrence of the optimum pH at 1.5 was due to some as yet unrecognized factor.

In order to investigate the influence of hydrogen ion concentration on protein synthesis, the pH was altered while other conditions were kept constant, and the resulting changes in the amount of protein synthesized were then observed.

Various amounts of acid and alkali were added and the resulting pH in each case ascertained electrometrically. The mixtures were then adjusted in such a manner that the dilutions were in each case the same, and the pH was again checked. The pH given in Table I is this final value. In these determinations of the hydrogen ion concentration, a Moloney electrode was employed (5).

From each mixture two 20 cc. portions were then pipetted into 50 cc. Erlenmeyer flasks. To each was added 1.0 gm. of pepsin (Eimer and Amend), which was dissolved in the digest by rubbing with a stirring rod. After adding 0.5 cc. of chloroform to each, the flasks were tightly stoppered and set away at 37.5°C. for 2½ days. They were then removed, neutralized with 50 per cent NaOH to stop further peptic action, diluted to 100 cc., and set away in an ice chest at 13°C. It was not possible to analyze any of the samples immediately, nor all of them simultaneously, so that the length of stay in the ice chest was not the same for all samples. The number of days intervening between neutralization and analysis is noted in the column headed "Time of standing" (Table II).

TABLE I.

Digest.	5 N HCl.	50 per cent NaOH.	H_2O	pH
cc.	*cc.*	*cc.*	*cc.*	
45	2.94	0.00	1.8	0.9
45	1.35	0.00	3.6	1.8
45	0.00	0.00	4.9	2.2
45	0.00	1.90	2.84	3.2
45	0.00	3.81	0.93	4.0
45	0.00	4.74	0.00	4.7

TABLE II.

pH	Series 1.		Series 2.	
	Time of standing.	Protein N in per cent of total N.	Time of standing.	Protein N in per cent of total N.
	days		*days*	
0.9	24	1.3	32	1.0
1.8	23	3.2	32	1.4
2.2	16	7.1	32	6.9
3.2	16	17.2		
4.0	11	27.3	28	23.3
4.7	11	18.7	28	16.0

The amount of protein synthesized was estimated by precipitation from the neutral diluted solution with trichloroacetic acid and is the difference between the total nitrogen contained in the solution before addition of the trichloroacetic acid and after filtering off the precipitate.

Very soon after dissolving the pepsin an increase in the opacity and viscosity was observed in the solutions. In the two most acid solutions (pH 0.9 and 1.8), the increase in turbidity was

only faint and the viscosity was not noticeably greater than in the control containing no enzyme (pH 4.0). The remaining solutions exhibited gradually increasing opacity and viscosity, progressively greater as the acidity of the solutions diminished. That at pH 2.2 was intermediate in opacity, 3.2 was densely opaque but still somewhat fluid, 4.0 and 4.7 were densely opaque and stiff immovable jellies. 2 hours later the same relationships were maintained, except that a slight precipitate had appeared in pH 1.8.

In Table II, Series 1 is the series first analyzed, *i.e.* which remained in the ice chamber the shorter period; Series 2 is the duplicate of Series 1, except that the samples were analyzed later.

Neither metaprotein nor trichloroacetic acid precipitable material other than insoluble protein could be detected in any of the experiments represented in Table II.

The optimum pH for protein synthesis in a peptic digest of egg albumin is at pH 4.0 (Tables II and IV). On standing at 13°C. for long periods some hydrolysis of the protein formed occurs. This is due not impossibly to a tissue enzyme which may be contained in the pepsin preparation and which is, as shown by Bradley (6), not killed at pH 2.0.

The figures obtained almost certainly represent equilibrium amounts and not velocities. This was clearly indicated in our earlier work, before the optimum hydrogen ion concentration had been discovered. Samples were set away at 38°C. at pH 1.7 for a number of days, and the synthesis followed from day to day (Table III). The concentrations of material were approximately the same, and the results are comparable with the results given above. It is clear that equilibrium is attained in approximately 2 days, even under suboptimal conditions.

The cause of the subsequent decrease shown in Table III is obscure.

A series was now carried out with most of the points on the alkaline side of pH 4.0. A concentrated, new peptic digest and Merck's pepsin, which gram for gram was found to be more potent than the pepsin previously used, were employed. The series was incubated for the usual period of 3 days at 38°C., after which the solutions were neutralized, diluted, and analyzed immediately for protein.

The optimum hydrogen ion concentration was again found to

be at pH 4.0; but the maximum amount of protein synthesized was higher than in the previous experiment. The results over the whole range are given in Table IV. They are the averages of duplicates whose maximum variation was of the order of magnitude of ±1 per cent of total nitrogen.

These figures, like those in Table II, represent equilibrium amounts. Mixtures incubated for 6 days showed no significant differences over those incubated for only 3 days; *e.g.*, one sample,

TABLE III.

Days.	Protein N in per cent of total N.
¾	3.3
1⅚	5.1
6⅚	4.4
10½	3.2
13½	2.4

TABLE IV.

pH	Protein N in per cent of total N.
2.8	20.2
3.4	31.0
4.0	33.7
4.3	31.9
4.45	32.1
4.9	30.7
5.45	13.6
5.6	13.9
6.0	5.9
6.6	3.3

similar to the mixture at pH 6.6 (Table IV), incubated for 9 days, gave 3.4 per cent of protein.

The percentages of the total nitrogen combined in the protein were calculated on the basis of the nitrogen content of the digest alone; *i.e.*, the added pepsin nitrogen was deducted from the total nitrogen of the mixture. The justification for this seemingly arbitrary procedure was found in the fact that a 24 per cent solution of pepsin at pH 4.0, maintained for weeks at 38°C., showed no signs of any formation of protein, notwithstanding that at the end of this period, both the synthesizing and hydrolyzing powers of the solution were found to have remained unimpaired.

In order to observe more easily the effect of acidity over the whole range in which protein synthesis occurs, the results in Tables II and IV were combined. These results, having been obtained on different digests and with different enzyme preparations, show considerable variation. They were therefore recalculated so that the maximum amount of protein synthesized in each case at pH 4.0 was taken as 100 per cent, and the lower amounts in each experiment as the corresponding fractions of this value (Table V). The resulting figures were plotted as ordinates, against the pH values as abscissæ.

TABLE V.

Protein Synthesized at Various Hydrogen Ion Concentrations.

pH	Actual value per cent of total N.	Per cent of maximum.
0.9	1.3	4.7*
1.8	3.2	11.7*
2.2	7.1	26.0*
2.8	20.2	60.0†
3.2	17.2	63.0*
3.4	31.0	91.9†
4.0	27.3	100.0*
4.0	33.7	100.0†
4.3	31.9	94.6†
4.45	32.1	95.3†
4.7	18.7	68.5*
4.9	30.7	90.1†
5.45	13.6	40.4†
5.6	13.9	41.3†
6.0	5.9	17.5†
6.6	3.3	9.8†

* Calculated on the basis of 27.3 per cent as maximum.

† Calculated on the basis of 33.7 per cent as maximum.

The curve obtained resembled in form and slope either the primary dissociation curve of a dibasic acid or the curve representing the undissociated residue of an amphoteric electrolyte.

To ascertain the degree of correspondence the curve was considered as such and the values for the primary and secondary dissociation constants, pK_1 and pK_2, or, in the alternative, the acid and basic dissociation constants of an ampholyte (pK_a and pK_b) were obtained by the tangent method of Michaelis.[1] These

[1] Michaelis (7), p. 64.

were found to be 2.67 and 5.33 for pK_1 and pK_2, and 5.33 and 11.33 for pK_a and pK_b.

The constants found were inserted into the formula given by Michaelis[2] and the degree of primary dissociation α and the magnitude of the undissociated residue ρ were calculated for pH intervals of 0.5. These calculated values, identical, of course, for α and ρ, are recorded in the first column of Table VI. In the second column of the same table the maximum value of α and ρ (91.4 per cent) is set as 100 and the other values readjusted to that scale and recorded as α_1 or ρ_1. This is done in order to facilitate a comparison with the observed amounts of synthesis obtained at

TABLE VI.

Values of α and ρ.

pH	Calculated α (ρ).	Modified α_1 (ρ_1).
1.0	2.1	2.3
1.5	6.6	7.2
2.0	17.6	19.3
2.5	40.3	44.1
3.0	68.0	74.4
3.5	86.0	94.1
4.0	91.4	100.0
4.5	86.0	94.1
5.0	68.0	74.4
5.5	40.3	44.1
6.0	17.6	19.3
7.0	2.1	2.3

various hydrogen ion concentrations which have also been expressed (Table V) as percentages of the maximum. On Fig. 1 a graphic comparison is obtained. The surprising approximation of the theoretical dissociation to the extent of synthesis is evident.

The hydrogen ion concentration in which primary ionization is a maximum is given by the equation $[H^+] = \sqrt{K_1 K_2}$. From pK_1 and pK_2, 2.67 and 5.33 respectively, the theoretical position of the maximum is at pH 4.0. Again, the isoelectric point of an ampholyte is given by the equation $I = \frac{\sqrt{K_a}}{K_b} K_w$ which, for $K_a = 5.33$ and $K_b = 11.33$, gives the isoelectric point at pH 4.0. This point is precisely the position of the experimental optimum.

[2] Michaelis (7), pp. 26 and 36.

The values for pK_1 and pK_2 do not correspond to the published dissociation constants of any of the known diamino or dicarboxylic acids, nor do the values for pK_a and pK_b correspond to the pub-

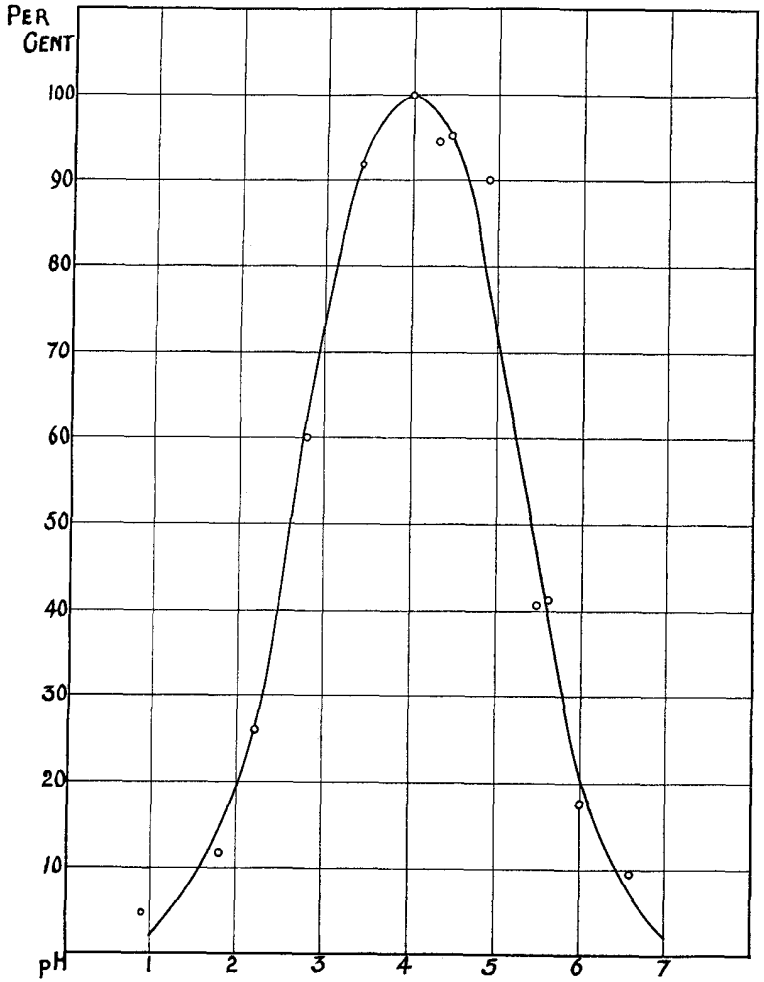

FIG. 1. Relation between pH (abscissæ) and amount of protein synthesized (ordinates). Curve represents calculated values of α_1 (or ρ_1) (Table VI). Points indicated o are the experimentally obtained protein values (Table V). Experimental points represent the average of two or more determinations.

lished dissociation constants of any amino acid. It is not impossible, however, that Fig. 1 may represent the dissociation of one of these amino acids as it exists in the concentrated digest, with the values for the constants somewhat modified by the presence or manner of its combination with other groups.

The importance of the above correspondence between the experimental values and the calculated dissociation curve rests in the conclusion drawn from it that the amount of protein synthesized at any given hydrogen ion concentration is dependent upon the degree of ionization of a component of the digest, *i.e.* the substrate, for electrometric titration curves showed quite unmistakably that the ionization of the digest, taken as an entity, was not represented by Fig. 1.

At the outset the effect of varying the hydrogen ion concentration might have been sought in variations brought about in the functional condition of the enzyme. There were two possibilities; the varying acidity affected either the rate of autodestruction or the ionization of the pepsin.

Assuming the first possibility, the optimum pH, then, is simply that degree of acidity in which synthesis proceeds to the greatest extent before it is brought to a stop by the disappearance of active enzyme.

The known facts regarding the autodestruction of pepsin do not support this hypothesis. Michaelis and Rothstein (8) and Morgenroth (9) have found that in 10 per cent NaCl, pepsin solutions at approximately pH 3.3 may stand at room temperature for a week without suffering any autodestruction, and in glycerol for more than a year. Our results are confirmatory, and it seems certain from extensive investigation in this laboratory that at acidities greater than pH 6.0 the autodestruction of pepsin cannot be a factor in determining the location of the optimum pH for peptic synthesis.

The alternative possible effect of acidity, *viz.* its influence upon the degree of ionization of the enzyme, remains.

In view of the previous result that the effect of pH on the amount of synthesis can be represented graphically by a dissociation curve, it follows, if the dissociation under consideration be the dissociation of the enzyme, that a direct linear proportionality must exist between concentration of active enzyme and extent of

synthesis. It is found, however, that the relationship between the equilibrium amounts of protein and the enzyme concentration is not linear, but exponential, of the type $Y = AX^n + B$.

We conclude that, in a broad sense, it is through the ionization of the substrate that the hydrogen ion exerts its influence on the peptic synthesis of protein. The steepness of the dissociation curve seems to preclude any possibility of accidental coincidence in the correspondence of the experimental values to the calculated curve. A similar explanation has been proposed by Northrop (10) for the location of the optimum pH of peptic hydrolysis.

That there should be any dependence of the equilibrium quantities of protein synthesized on the concentration of the pepsin was a surprising result, though indications of the same phenomenon are to be found in the experimental results of Sawjalow (3) and of Robertson (11).

TABLE VII.

Digest No.	0.1 HCl added.	Enzyme (Merck).	Concentrated digest at pH 4.0.	Total N.	N after filtration.	Protein N.	Protein N in per cent of total N.
	cc.	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*	
1	1	0.01	10	857	744	113	13.1
2	1	0.05	10	848	681	167	19.7
3	1	0.10	10	858	661	197	23.0
4	1	0.20	10	843	615	228	27.1
5	1	0.40	10	837	584	253	30.1
6	1	0.80	10	839	560	279	33.2

In attempting to determine this effect of enzyme concentration on the extent of synthesis the procedure adopted was the same as that used in the previous experiments. Six mixtures were made with relative enzyme concentrations varying over a range from 1 to 80. The pH and the concentration of the digest were unchanged. The full details and the results are given in Table VII.

Enzyme action was allowed to continue for 36 hours at 37°C. The samples were then neutralized, diluted to 100 cc., and analyzed for protein in the usual manner. The figures for total nitrogen are the values for the digest alone, *i.e.* the nitrogen content of the added pepsin subtracted from the figure actually obtained.

The curve for protein formation plotted against enzyme concentration is of the type of $Y = AX^n + B$. When $X = 0$, $Y = 0$;

therefore $B = 0$. Average values for A and n are 13.9 and 0.213 respectively, and the equation becomes $Y = 13.9\ X^{0.213}$.

The curve in Fig. 2 is the calculated curve, and the indicated points represent experimental values.

The above result is not in itself capable of any definite interpretation. Presumably the figures for protein represent equilib-

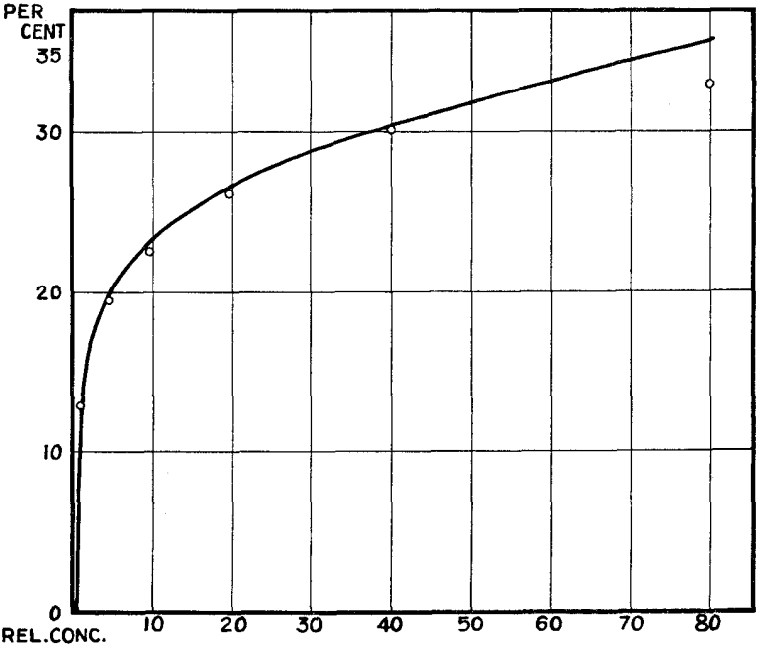

FIG. 2. Relation of pepsin concentration (abscissæ) to equilibrium amount of protein synthesized (ordinates). Curve drawn from empirical equation $Y = 13.9\ X^{0.213}$. Points o represent experimental values (data in Table VII).

rium amounts, because the highest concentrations of enzyme employed were relatively enormous, 1 to 8 per cent. Even the lowest, 0.1 per cent, is more than adequate for the complete hydrolysis of 6 per cent albumin in 36 hours, if the pH is maintained at the optimum. To decide the point, the amount of protein synthesized with a low enzyme concentration, 0.5 per cent, was followed over a fairly lengthy period, and the time noted for the

attainment of equilibrium. Two other experiments were also carried out under identical conditions, except that in one case 5 per cent pepsin (Eimer and Amend), and in the other 8 per cent pepsin (Merck) was employed. The results of these experiments are shown in Table VIII.

With 0.5 and 5.0 per cent pepsin, equilibrium is apparently reached in about 36 hours. With greater enzyme concentrations, larger amounts of protein are formed.

If the true equilibrium position of the system can be represented by the final amount synthesized, then we have here an exception to the general law of enzyme action and catalysis, which states that the equilibrium position is not affected by the concentration of the enzyme.

TABLE VIII.

0.5 per cent pepsin (Merck).		5.0 per cent pepsin (Eimer and Amend).		8 per cent pepsin (Merck).	
Time.	Protein N in per cent of total N.	Time.	Protein N in per cent of total N.	Time.	Protein N in per cent of total N.
hrs.		*hrs.*		*hrs.*	
6	9.1				
18½	14.1				
24	16.6	24.0	25.0	24.0	33.2
36	18.6				
48	18.6				
		60.0	27.3		

SUMMARY.

1. The optimum C_H for protein synthesis in peptic digests of egg albumin is at pH 4.0.

2. The hydrogen ion concentration exerts its influence through the ionization of some unidentified di-acid or amphoteric constituent of the digest.

3. Under the conditions in which protein synthesis occurs, there is no appreciable autodestruction of pepsin.

4. The amount of protein formed is partially dependent upon the concentration of pepsin.

5. The quantitative aspect of this relationship precludes the possibility that ionization of the enzyme influences the location of the optimum hydrogen ion concentration.

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