

THE ENZYMATIC SYNTHESIS OF PROTEIN. II.

THE EFFECT OF TEMPERATURE ON THE SYNTHESIZING ACTION OF PEPSIN.

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In a solution of the products of the hydrolysis of protein it is theoretically possible to bring about the reverse reaction, *i.e.* synthesis, in two ways: by concentrating the solution, and by raising the temperature. The theoretical considerations from which the first of these conclusions was deduced have been discussed in a previous paper (1). It is sufficient to recapitulate here, that the first method is predictable from an appropriate statement of the mass law. The experimental confirmation of the prediction was described by the authors (1). The second method is predictable from certain thermodynamical considerations of reversible reactions pointed out by Moore (2). He deduced the equilibrium equation $P_a = K P_b^n$, where P_a and P_b are respectively the osmotic pressures of the substrate and its product, and K is a constant. K is a symbol for the expression $P_0 e^{\frac{C}{RT}}$, where P and e are constants, R is the gas constant, C is the chemical energy involved in the breakdown of 1 gram molecule of A into n gram molecules of B , and T is the absolute temperature.

As Moore points out, it is obvious, from an inspection of the functions combined in the value of K , that an increase of temperature should have the reverse effect of an increase in the value of C .

A large value for C obtains always in reactions where reversibility is difficult of attainment. It follows that, especially in those reactions where C has neither a very high positive nor a very high negative value, an increase of temperature should

facilitate reversibility. In such cases, at high temperatures, synthesis may be possible where, at low temperatures, hydrolysis was in progress.

The value of C for the hydrolysis of proteins is practically zero (3). Protein hydrolysates present, therefore, almost an ideal system for testing the effect of temperature on reversibility.

EXPERIMENTAL.

A 6 per cent solution of egg albumin (Merck) was digested with 0.1 per cent pepsin (Merck) at pH 1.7 for 7 days. At the end of this period it gave no precipitate with either trichloroacetic or copper acetate, indicating that protein and primary proteose were absent. It was filtered, adjusted to pH 4.0, and without increasing the concentration, or adding any more enzyme, a portion was heated to 65°C. In 10 minutes a precipitate appeared, and in $\frac{1}{2}$ hour a large amount was obtained. This was filtered off, thoroughly washed, and dissolved in 0.025 N NaOH. A biuret test was carried out, and a typical protein color was obtained, while the color given by the digest maintained at 20°C. was the rose shade of proteose and peptones. Another smaller amount of this precipitate was dissolved in 0.025 N HCl, and, on the addition of trichloroacetic acid, a characteristic precipitate of plastein or native protein appeared. The solution at pH 4.0, maintained at 24°C., showed no precipitate, and remained perfectly clear over a period of 8 hours. Boiling the digest and then maintaining at 65°C. for 8 hours brought no increased precipitation over the faint coagulation produced on boiling. Some of the original solution was adjusted to pH 1.7, instead of to pH 4.0 (introducing the same dilution as in the adjustment to pH 4.0), and was then maintained at 64°C. for 6 hours. Only a slight precipitate was obtained, unlike plastein in appearance, and too slight to be filtered. This precipitate may have been protein, because a slight amount of synthesis is possible at pH 1.7.

We have shown (1) that the insoluble material formed under such conditions is a product synthesized from the components of the digest, and that it is of the complexity of protein. We feel justified, therefore, in referring to this product in future, simply as protein.

The effect of temperature is illustrated in another type of experiment.

0.5 gm. of pepsin (Merck) was dissolved in 0.1 N HCl in each of eight flasks, brought to the temperature indicated in Table I, and maintained there for $\frac{1}{2}$ hour. 10 cc. of a concentrated solution of the products of peptic hydrolysis, at the same temperature and at pH 4.0, were added, thoroughly mixed, and set away with chloroform at this temperature for 24 hours. At

the end of this period the flasks were removed, their contents neutralized to stop any further peptic action, and diluted to 100 cc.

The resulting mixtures were then analyzed for the insoluble protein by estimating the total nitrogen before and after filtration.

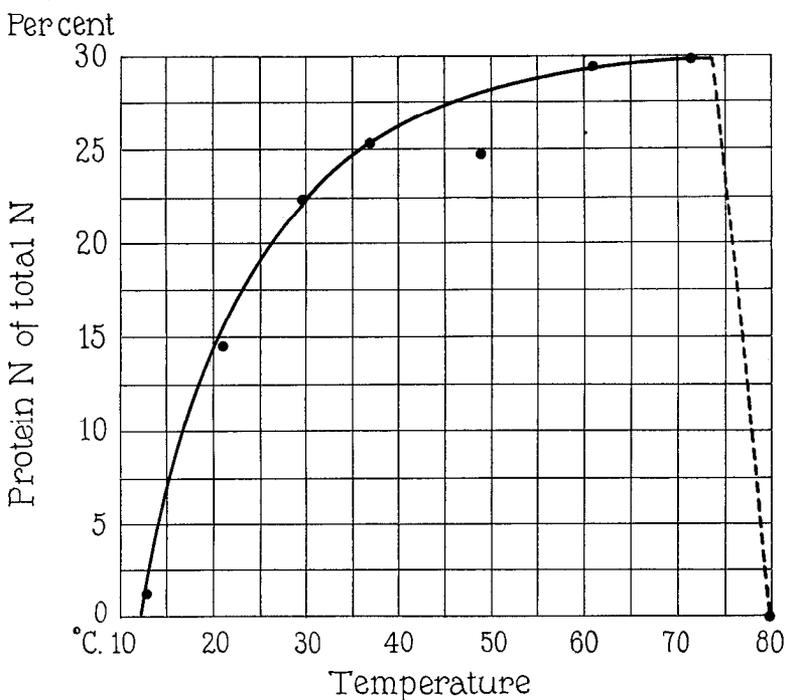


FIG. 1. Effect of temperature on the amount of protein formed.

As will be shown in a later paper, with the possible exception of the lowest temperature, these represent, in all probability, close approximations to equilibrium amounts. The effect of increase of temperature, then, is to move the equilibrium point more and more over to the protein side, *i.e.* to increase the possibility of reversion (Fig. 1).

There seems to be no necessity for postulating, as Robertson (4) does, two enzymes or two components of one enzyme, one functioning in hydrolysis and the other in synthesis. Assuming only the classical function of a catalyst, a shift in equilibrium is accounted for on thermodynamic grounds, when the only influence ascribed to the enzyme is the acceleration of the attain-

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ment of the point of equilibrium, wherever that point may be defined by the heat, chemical energy, and osmotic pressures of the reacting components.

Inseparability of the Hydrolyzing and Synthesizing Components of Pepsin.

Specially designed experiments showed that the processes of boiling and of addition of alkali, which destroyed the hydrolyzing protease pepsin, also resulted in the destruction of the synthesizing power of the commercial pepsin preparation.

TABLE I.
Effect of Temperature on Peptic Synthesis.

Temperature.	Total N.	N in filtrate.	Protein N.	Protein N of total N.
°C.	mg.	mg.	mg.	per cent
13	818	809	11	1.5
21	811	693	118	14.5
30	828	642	186	22.4
37	397	297	100	25.3
49	830	824	206	24.9
61	806	568	238	29.5
72	823	576	247	30.0
80	823	823	0	0

Four flasks, Nos. 1, 2, 3, and 4, were set up. In Flask 1, 1.00 gm. of pepsin (Merck) was dissolved in 2 cc. of 0.1 N HCl, giving clear solution, and was set away at 37°C. for 1 hour. The solution remained clear. 20 cc. of concentrated peptic digest at pH 4.0 were pipetted in, and thoroughly mixed. Chloroform was added and the flask was tightly stoppered and set away at 37°C. for 24 hours. In 10 minutes a precipitate appeared in the clear solution, and in 4 hours the formerly quite fluid digest was transformed into a solid opaque jelly. In Flask 2, 1.00 gm. of pepsin (Merck) was dissolved in 2 cc. of 0.1 N HCl and set away at 37°C. for 1 hour. The flask was then placed for 15 minutes in boiling water and for $\frac{1}{2}$ minute on a hot-plate. The solution was coagulated. It was then cooled and thoroughly mixed with 20 cc. of concentrated digest. Quite unexpectedly the coagulum dissolved, leaving only a slight turbidity which did not increase on standing 24 hours at 37°C. In Flask 3, 1.00 gm. of pepsin was dissolved in 1 cc. of 1.0 N NaOH, alkali of this concentration being required to render the solution alkaline to litmus. A precipitate appeared immediately. It was set away at 37°C. for 1 hour and then neutralized with 1.5 N HCl so that the solution became acid to litmus. 20 cc. of the concentrated peptic digest were added. After

thorough stirring, this precipitate also dissolved, leaving a slight turbidity which did not increase during the 24 hours standing at 37°C. Flask 4 was identical in contents with Flask 3.

At the end of 24 hours, the contents of Flasks 1, 2, and 3 were neutralized, diluted to 250 cc., and analyzed for protein. The contents of Flask 4 were used for the estimation of the pH, which was found to be 4.0. The results of the analysis are given in Table II.

457 mg. of protein nitrogen were formed in Flask 1, none in Nos. 2 or 3. The turbidity of the coagulated enzyme yielded little or no nitrogen, a surprising result, which has been confirmed on a number of occasions. The results are unequivocal; boiling and alkali destroy the synthesizing power of commercial pepsin. The statement of Bayliss (5) that alkali, which destroys the

TABLE II.
Effect of Boiling and Treatment with Alkali on the Synthesizing Power of Pepsin.

	Flasks.		
	1 Normal pepsin.	2 Boiled pepsin.	3 Alkali- treated pepsin.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Total N before filtration.....	1,802	1,752	1,789
“ “ after “	1,345	1,752	1,785
Protein N.....	457	0	4(?)

hydrolytic activity of pepsin, does not remove the precipitating power, is not applicable to our experiments. Bayliss, in discussing Robertson's results, suggests that the synthesis (precipitation), described by him, was probably due to the particular sample of pepsin employed. It seems possible that the synthesis obtained by Robertson is similar to that known as plastein formation, and it may therefore be worth pointing out that protein (plastein) has been obtained by Danilewski and other Russian workers with stomach extract. Robertson employed Grubler's pepsin, and we have employed Eimer and Amend's pepsin and two batches of Merck's.

Further, to remove another objection by Bayliss, the acid peptic hydrolysates utilized by us were in every case filtered be-

fore concentration, and this in no way prevented the formation of protein in our digests of egg albumin.

So far no means of separating the hydrolyzing and synthesizing components had presented itself. Treatment which destroys one also destroys the other. But in view of the statement found in the literature (Bayliss (5) and Robertson (4)) that the hydrolyzing component of pepsin is destroyed at 65°C., a method seemed to be available. At 72°C. the maximum yield of protein was obtained, and if heating at 65°C. under the conditions of protein formation destroys the hydrolyzing power of pepsin, the dissimilarity of the synthesizing and hydrolyzing components is proven.

TABLE III.
Effect of Temperature on the Hydrolyzing Component of Pepsin.

Flask No.	Time.	Free amino nitrogen.
	<i>hrs.</i>	<i>mg.</i>
	0	0.62
1	27	2.97
2	27	2.47
3	27	0.99

In each of three flasks, Nos. 1, 2, and 3, 0.4 gm. of Merck's pepsin was dissolved in 1 cc. of HCl, the resulting pH being 3.3. No. 1 was maintained at 60°C. for $\frac{1}{2}$ hour; No. 2 at 72°C. for $\frac{1}{2}$ hour; and No. 3 at 80°C. for $\frac{1}{2}$ hour. At the end of this period Nos. 1 and 2 were quite clear, and No. 3 was coagulated. 50 cc. of 3 per cent albumin at pH 1.7 were added to each, and the flasks tightly stoppered and set away with chloroform at 37°C. Free amino nitrogen estimations were carried out on 5 cc. of the solution immediately, and 27 hours later. The results are given in Table III.

Flasks 1 and 2, after 27 hours, gave turbidities only, when trichloroacetic acid was added, while No. 3 gave a voluminous, flocculent precipitate. The slight increase in the amino nitrogen in No. 3 was probably due to the hydrolyzing effect of the acid.

The results of this experiment indicate that temperatures of 60°C. and 72°C., maintained for $\frac{1}{2}$ hour, do not destroy the hydrolyzing power of pepsin. Robertson, and also Bayliss, may have overlooked the possibility of the protective influence of concentrated solutions of the products of hydrolysis on the enzyme.

Our previous experiment proved that the processes of boiling and addition of alkali destroy not only the hydrolyzing, but also the synthesizing properties of our pepsin preparation. The last experiment proves that maintenance at a temperature of 72°C. which gives a maximum synthesis does not, as might have been supposed, destroy the hydrolyzing properties, and the only conclusion possible is that the hydrolyzing and synthesizing components are inseparable and are in all probability identical. The higher yield of protein at 72°C. is predictable without postulating any influence of temperature upon the condition of the catalyst. The action of the latter is to accelerate the attainment of equilibrium wherever that point is defined by the temperature and the concentration, whether on the side of hydrolysis or of synthesis.

SUMMARY.

1. In concentrated peptic hydrolysates of protein, at pH 4.0, in the presence of pepsin, increasing amounts of protein are formed with increasing temperature, up to the point of destruction of the enzyme.

2. This result is shown to be predictable from certain thermodynamical considerations of reversible reactions.

3. The synthesizing and hydrolyzing components of commercial pepsin were found to be inseparable.

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