THE ENZYMATIC SYNTHESIS OF PROTEIN. IV.

THE EFFECT OF CONCENTRATION ON PEPTIC SYNTHESIS.

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(Received for publication, February 14, 1925.)

In the enzymatic hydrolysis and synthesis of proteins *in vitro*, the important factor, the factor upon which the direction and the degree of the reaction are dependent, is not the relative concentration of water, but the concentration of material in solution. This conclusion, pointed out by Moore, the authors have discussed at length in a previous paper (1). As shown there, the molecular concentration of water is always so enormously greater than that of the other components that the small amounts added or removed in the course of either reaction are negligible, and it may, therefore, be considered as remaining constant. The distinguishing feature of the hydrolysis and synthesis of protein is the conversion of 1 molecule of protein into a number of molecules of products. It is this characteristic which is responsible for complete hydrolysis in dilute solutions and for the ease with which synthesis is achieved in concentrated solutions. It follows that the extent of synthesis will increase as the concentration increases, and that as the concentration decreases a point will be reached at which synthesis will fail. The concentration at this point will correspond to the maximum concentration of protein capable of complete hydrolysis.

To locate this crucial concentration, two series of experiments were designed. In both series, the amount of protein synthesized in various concentrations of hydrolytic products was compared, but in one series (*a*) the enzyme concentration varied with the concentration of products, in the other (*b*) the enzyme concentration was kept constant. The arrangement of conditions in Series *a* and the results obtained are shown in Table I.

The mixtures, consisting of various dilutions of a peptic hydrolysate of albumin to which pepsin had been added, were preserved with chloroform and were contained in stoppered Erlenmeyer flasks. They were set away at 33°C. for 48 hours. After 48 hours the solutions were neutralized and diluted to 250 cc., and the total nitrogen estimated in duplicate on 25 cc. of the suspension. The protein was filtered off and the total nitrogen estimation again carried out on 25 cc. of the filtrate. The difference between the two was the amount of protein nitrogen.

In 10 minutes at room temperature heavy precipitates appeared in Dilutions 1 and 2 and a slight precipitate, in 3. The other three dilutions showed no noticeable increase in turbidity. After 24 hours at 33°C. a slight precipitate had appeared in 4, while

TABLE I.

Series a.

Effect of Substrate Concentration on Synthesis with Varying Enzyme Concentrations.

Digest at pH 3.9.	Pepsin.	0.0001 N HCl.	Dilution.	N in 100 cc.	Total N.	N in filtrate.	Protein N.
cc.	*gm.*	*cc.*		*mg.*	*mg.*	*mg.*	*mg.*
20	1.01	0	1	6,500	1,300	1,100	200
20	1.01	20	2	3,270	1,310	1,210	100
20	1.02	40	3	2,230	1,340	1,280	60
20	1.01	80	5	1,310	1,310	1,300	10
20	1.00	130	7.5	870	1,300	1,300	0
20	1.00	180	10.0	650	1,310	1,310	0

the protein precipitates in 1, 2, and 3 had become denser; 5 and 6 were still without protein precipitates after 48 hours; and the amount of protein precipitated in 1, 2, 3, and 4 varied visibly, inversely as the dilution. The pH of 4, 5, and 6 was 3.9 at the end of the experiment.

Up to the fifth dilution the amount of protein is inversely proportional to the dilution. The concentration at which no protein formation occurs, at 33°C., lies between 870 and 1,310 mg. of nitrogen per 100 cc.

The arrangement of conditions and the results obtained with Series *b* are shown in Table II.

In all dilutions except 8 and 10 (Table II) the typical protein precipitates appeared in less than 10 minutes. The flasks were

set away at 37°C. for 48 hours; they were then neutralized, diluted, and analyzed for protein in the usual manner. The pH of 7 and 8 at the end of 48 hours was 4.0.

Protein synthesis again ceased at a concentration approximating that of the previous experiment, *viz.* 1,270 mg. per 100 cc., despite an 8-fold increase in the enzyme concentration. This value corresponds to approximately 8.0 per cent of protein. The concentration of protein in plasma is from 7 to 8 per cent. While it is not possible at present to infer any direct connection between these two facts, the similarity in value seems possibly significant.

The results of the two series of experiments (*a* and *b*), cannot be combined in one set of data. Series *a* was carried out at 33°C.

TABLE II.

Series b.

Effect of Substrate Concentration on Synthesis, with Enzyme Concentration Constant.

Digest at pH 3.9.	Pepsin.	0.0001 N HCl.	Dilution.	N in 100 cc.	Total N.	N in filtrate.	Protein N.
cc.	*gm.*	*cc*		*mg.*	*mg.*	*mg.*	*mg.*
10	0.4	1	1	8,050	890	680	210
10	0.8	12	2	4,280	940	800	140
10	1.2	23	3	2,990	990	910	80
10	1.6	34	4	2,360	1,040	990	50
10	2.0	45	5	2,030	1,120	1,070	50
10	2.4	56	6	1,690	1,170	1,130	40
10	3.2	78	8	1,430	1,260	1,250	10
10	4.0	100	10	1,270	1,400	1,400	0

or 4° lower than Series *b* and with a brand of pepsin which gram for gram was less active than that used in Series *b*. The same general conclusions may, however, be drawn quite definitely from both; a straight line relationship between the amount of protein synthesized and the concentration of material in solution (Fig. 1), a falling off from the straight line relationship at high concentrations, and approximately the same minimum concentration for protein formation.

The falling off from the straight line relationship with the higher concentration of material is definite in both, and the same phenomenon to a more marked degree, appeared in experiments designed to effect 100 per cent synthesis.

A mixture of enzyme and digest under optimal conditions (*i.e.*, pH 4.0; 4.0 per cent pepsin and concentrated hydrolysate) was set away in a vacuum desiccator at 38°C. A sufficiently low pressure was maintained to cause the digest to froth into large bubbles. A very great concentration of products and a great diminution in

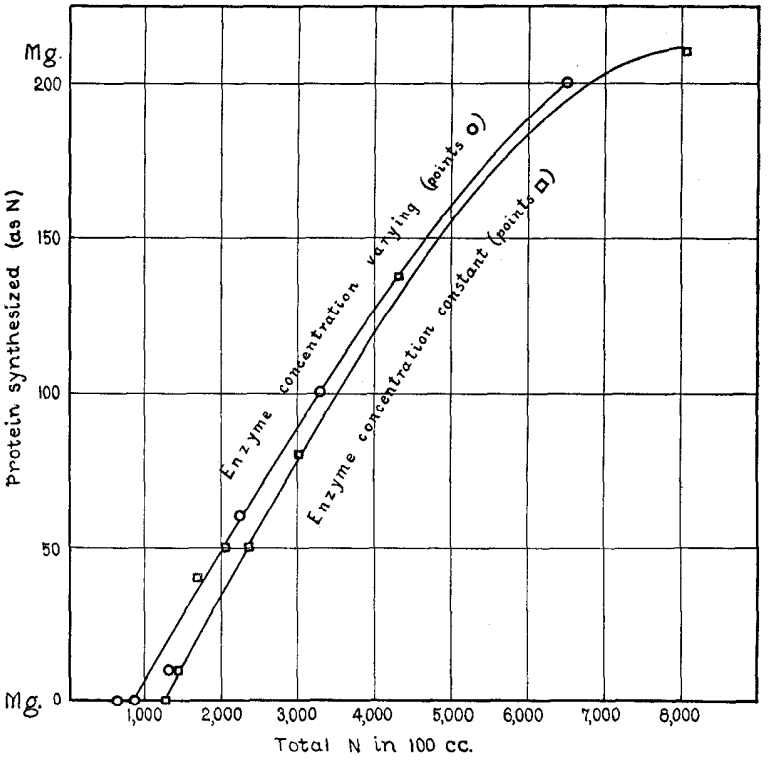

FIG. 1. Relation between amount of protein synthesized and the concentration of material in solution.

the water content was thus achieved, and the mixture was practically dry so that a greatly increased yield may justifiably have been expected. To our surprise, analysis after 48 hours showed only traces of protein to have been formed. In very great concentrations of material not only had there been a relative falling off in the amount of protein, but also a large absolute decrease.

Persisting still in the endeavour to obtain a large yield, we dissolved 0.5 gm. of pepsin (Merck) in 10 cc. of 0.01 N HCl; 10 cc. of concentrated peptic digest at pH 4.0 were added, with chloroform, and the mixture was set away at 37°C. for 3 hours. By that time the digest had become a solid jelly, with the whitish yellow colour indicative of the formation of large amounts of protein. The flask was now uncorked and set in a sulfuric acid desiccator under a slightly diminished pressure, at 38°C. 21 hours later, the vacuum was greatly increased to a pressure of 50 mm. of mercury and the desiccator replaced at 37°C. By diminishing the pressure thus gradually, it was hoped that the evaporation of water from the digest would be slow enough to allow the protein formation to proceed beyond the limits yet attained, before the inhibiting effect of a too low concentration of water could overcome the synthesizing tendency of the increased concentration of products.

At the end of 48 hours the flask was removed and analyzed for protein and 30 per cent was found. The amount of protein produced by 0.5 gm. of pepsin, at 38°C. in 36 hours, without additional concentration, was found to be 31 per cent. Disregarding the difference of 1 per cent, it may be concluded that further concentration of the solution, even to a relatively slight degree, was without any augmenting effect on the amount of protein formed.

A related phenomenon was encountered by Armstrong and Gosney (2) in their experiments on the enzymatic synthesis of fats. They attributed the inhibitory influence of too low concentrations of water to the resulting diminution or removal of the surfaces in the heterogeneous systems of oily substances in water with which they were dealing. In their own words: "Apparently, as pointed out by us previously, the intervention of a film of water is necessary at the interface of the system, where interaction takes place; if this be removed, action comes to an end." This explanation will not suffice here, because all the reacting substances, except the precipitated protein, are in solution.

Another more serious problem arose from the practical coincidence of the minimal concentrations of digest for protein formation at the identical point, with enzyme concentrations varying as widely as 4 and 0.5 per cent. If the minimal concentration of products necessary for synthesis at 38°C. is at approximately 8 per cent, in view of the insolubility of protein it would be expected

that synthesis would proceed at all higher initial concentrations until the concentration had fallen to the critical value of 8 per cent.

Table III shows that the facts are otherwise.

None of the factors which are usually considered to account for the cessation of an enzyme action, appear to be functioning here. All the available substrate was not utilized, or the same amount of protein would have been formed in each; the enzyme concentration was maintained constant in Series *b*, so that variation in the amount of enzyme, which has been previously shown (3) to affect the equilibrium, cannot be the cause; the possibility of autodestruction of the enzyme has been ruled out experimentally, and previous work had shown that the above figures represent equilib-

TABLE III.

Amount of protein formed in 100 cc.	Concentrated substrate in 100 cc. before protein formation.	Concentrated substrate in 100 cc. after protein formation.
gm.	*gm.*	*gm.*
11.9	50.3	38.4
3.9	26.7	22.8
1.5	18.7	17.2
0.7	14.7	14.0
0.5	12.7	12.2
0.4	10.6	10.2
0.1	8.9	8.8
0.0	7.9	7.9

rium amounts and not velocities (3). The speed of protein precipitation was visibly greater in the more concentrated solutions, where the discrepancy between the critical 8 per cent and the final concentration is greatest.

Two possible explanations remain. Either, in the synthesis of protein, enzyme is removed, or the figures represent equilibrium amounts in the specific mass law sense of the term equilibrium. If the latter be true, initial addition of protein, even though, as in this case, insoluble, should inhibit the subsequent synthesis of protein from the digest; and the extent of the inhibition should be proportional to the amount added.

The following experiment was devised in an attempt to test the first possibility. 0.5 gm. of pepsin was dissolved in 0.0001 N

HCl; 7.5 cc. of digest at pH 4.0 and 0.5 cc. of chloroform were added; the flask tightly stoppered, and set away at 37°C. 50 hours later an additional 7.5 cc. of digest were pipetted in, thoroughly mixed with the heavy gelatinous paste of digest and protein already present, tightly stoppered, and set away again at 37°C. This was Flask *A*. Another flask, *B*, containing 0.5 gm. of pepsin and 15 cc. of digest, was incubated for the same total length of time as was *A*. During the period of incubation the mixtures were thoroughly stirred twice in each 24 hours.

After 5 days Flasks *A* and *B* were removed from the incubator and the contents analyzed for protein.

Assuming that enzyme is removed in the course of synthesis; it follows that the total amount of protein formed in *A* will be less than in *B*, because the enzyme, which has promoted the formation of protein in the first portion of digest in *A*, will have been in large part removed. The percentage of synthesis in the second portion will consequently have been low, and presumably the sum of the synthesis in the two portions should be less than if the whole of the enzyme had been allowed to act upon both portions of the digest at once as in *B*. It is probable, of course, that the percentage of protein formed in the first portion of *A* will be slightly greater than would be formed in the whole digest of *B*, on account of the greater concentration of enzyme, but it would also probably not be great enough to compensate for the large falling off in the second portion.

If, on the other hand, there is no difference between *A* and *B*, then we have, to say the least, no evidence that enzyme has been consumed. On analyses, the latter result was obtained.

The amounts synthesized in *A* and *B* were 410 and 402 mg. of protein nitrogen respectively, or 39.3 and 38.3 per cent of the digest N. Practically the same amount of protein was synthesized in each flask and we have no evidence of enzyme removal in the progress of synthesis.

It is to be admitted that this experiment is not conclusive, but it was impossible to devise one less unsatisfactory, and fortunately the second possibility, *viz.* that addition of protein will inhibit synthesis, proved to be readily demonstrable, and it is therefore unnecessary to explain the results by the removal of enzyme.

In a tube, *C*, a suspension of boiled and thoroughly washed

synthesized protein, containing 146 mg. of nitrogen, was centrifuged. The supernatant fluid was carefully poured off, leaving the solid heavy precipitate in the tube. 0.5 gm. of pepsin and 3.5 cc. of 0.0001 N HCl were added, the enzyme was dissolved, and then 12.5 cc. of concentrated peptic digest pipetted in. The mixture was thoroughly stirred, and set away at 37°C. A flask, *D*, containing 0.5 gm. of pepsin, 12.5 cc. of digest, and 2.5 cc. of 0.0001 N HCl, was prepared and set away at 37°C. at the same time as was *C*. During the period of incubation the mixtures were thoroughly stirred twice in each 24 hours. After 5 days the flasks were removed from the incubator and their contents analyzed for protein.

If the added protein, though not in solution, is nevertheless a component of the system in equilibrium, then the amount synthesized in *C* will be less than in *D*. The results showed this to be the case. In *C*, where protein equivalent to 146 mg. of N had been added, protein equivalent to only 266 mg. or 29.9 per cent of the digest N was synthesized, while in *D*, where no protein was added, protein equivalent to 302 mg. of N or 35.7 per cent of the digest N was synthesized.

The insoluble added protein exerted an inhibiting influence on the synthesis of protein from the digest. Though not in solution it acted as if it were in solution. The total amounts of nitrogen in suspension, and of protein present at equilibrium, in *A* and *C* were identical. The protein, though precipitated, retains the same significance as a compound in solution in its influence upon the condition of equilibrium.

This unexpected conclusion was submitted to a more rigid verification.

Five flasks were prepared as in the previous experiment, and various amounts of thoroughly washed and boiled freshly synthesized protein were added. Water and digest were added in such amounts that the nitrogen concentrations of the suspensions were the same in all. The contents of the flasks are tabulated in Table IV, and the results in Table V. They were incubated at 37°C. for 4 days and then analyzed for protein.

In the last column of Table V are given the lesser amounts of protein that would have been formed in the absence of added protein as a result merely of the dilution introduced in adding the protein.

The figures in the last two columns remove the possible objection that the dilution is the cause of the lower amounts synthesized in the mixtures to which synthesized protein had been added; and the definite result is obtained that the extent of synthesis is less in those mixtures containing added protein than can be accounted for by dilution.

The results in the third and fourth columns strikingly confirm the conclusion drawn from the previous experiment. In Fig. 2 the

TABLE IV.

Digest.	Pepsin.	H_2O	Protein N.
cc.	*gm.*	*cc.*	*mg.*
10	0.4	1.0	0
9.5	0.4	1.5	39
9	0.4	2.0	77
8	0.4	3.0	154
7	0.4	4.0	231

TABLE V.

Total N.	N after filtration.	Protein N.					
		Added.	Synthesized.	Added and synthesized.		Synthesized.	Hypothetical synthesis.
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent of total*	*per cent of digest N*	*per cent of digest N*
794	570		224	224	30.2	30.2	30.2
829	595	39	195	234	30.1	26.4	28.7
836	593	77	166	243	31.0	23.5	27.1
807	530	154	123	277	36.6	20.5	24.6
777	474	231	72	303	41.8	14.6	21.1

amounts of protein synthesized are plotted as ordinates against the protein added as abscissæ. They give a steep, straight line.

One unexplained result, however, does remain among those given in Table V. This is shown in Fig. 2 by the failure of the straight line to cut the horizontal axis at an abscissa equal to the ordinate at which it cuts the vertical axis. It would seem that the addition of synthesized protein to a solution of enzyme and digest does not retard subsequent protein formation as much as the synthesis itself.

Addition of a solution of egg albumin to a solution of digest and pepsin inhibited the synthesis of protein in the same manner as synthesized protein itself. The amount of albumin that could be added without diluting the solution too greatly was, however, small, and in addition the albumin was denatured and precipitated as soon as it was mixed. The result, therefore, carries no additional significance beyond confirming the phenomenon already observed; *i.e.*, the inhibition of a reaction occurring between sub-

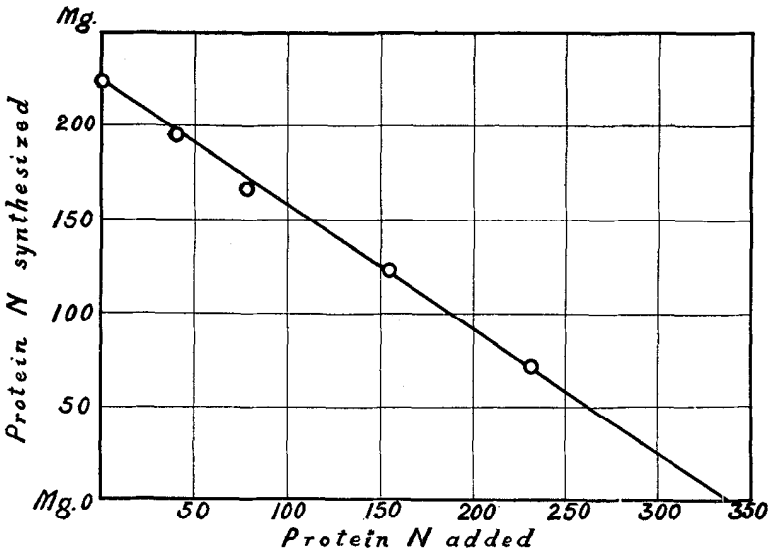

FIG. 2. Relation between the amounts of protein synthesized and the amounts of presynthesized protein added.

stances in solution, by the presence of material, which in the usual sense, is insoluble in that solution.

The above experiments showed that in peptic hydrolysates of egg albumin, in the presence of pepsin, more and more protein is synthesized as the concentration increases above a value corresponding to approximately 8 per cent of protein. Considering the whole nitrogen content as having arisen from the protein present, this conclusion can be restated; that, as the initial concentration of protein increases the degree of hydrolysis decreases. This is

experimental confirmation of a prediction made on theoretical grounds in a previous paper (1). The proof, however, is indirect.

An experiment was carried out to obtain, if possible, direct confirmation. A difficulty was encountered, however, which at present seems insuperable. Solutions of albumin of greater concentration than 8 per cent, in acidities as high as pH 2.0, become jellied and coagulated. On subsequent dilution only part of the coagulum redissolves, and if one attempts to filter the jelly, the bulk of the protein remains on the filter, so that the filtrate is only a dilute solution of protein. This gelatination does not occur with 6 per cent albumin; nor even with solutions as highly concentrated as egg white, *viz.* 14 per cent, when the reaction is neutral or slightly alkaline.

Concentrated solutions of albumin were submitted to peptic hydrolysis at pH 1.6 for a number of days and the degree of hydrolysis noted at intervals of 24 hours. The results obtained have at best only qualitative value; but their tendency is in accord with the predictions made. A 6 per cent solution of albumin was completely hydrolyzed, *i.e.* it contained no demonstrable protein, in 3 days at 37°C. A solution of egg white, 11.2 per cent, freed from membrane, was only 53 per cent hydrolyzed; a 17.4 per cent solution was 44.5 per cent hydrolyzed. In the latter two solutions no change was observed after 48 hours, though the pH was maintained at 1.6. During the first 48 hours, the pH continually rose, as is usual in peptic hydrolysis; and required continual adjustment. After that, for the next 2 days, it remained unchanged.

SUMMARY.

1. In a solution of the products of peptic hydrolysis of albumin, the extent of synthesis with pepsin is in simple inverse proportionality to the dilution.

2. This relationship does not obtain in very concentrated solutions, and in sufficiently high concentrations the amount of synthesis is actually less than in more dilute solutions.

3. With enzyme concentrations varying between 4.0 and 0.05 per cent, synthesis fails at 38°C. in a solution of products which corresponds to approximately 8 per cent of protein.

4. No evidence for the possibility that enzyme disappears in the course of synthesis was obtained.

5. Addition of synthesized protein to a solution of digest and pepsin inhibits the subsequent synthesis to an extent directly proportional to the amount added.

6. A similar inhibition of synthesis also occurs on the addition of native protein.

7. Peptic hydrolysis similarly does not proceed to completion in concentrated protein solutions.

BIBLIOGRAPHY.

1. Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, 1924–25, lxii, 15.
2. Armstrong, H. E., and Gosney, H. W., *Proc. Roy. Soc. London, Series B*, 1914–15, lxxxviii, 176.
3. Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, 1924–25, lxii, 675.