THE SUBSTRATE IN PEPTIC SYNTHESIS OF PROTEIN

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Pepsin exhibits in its proteolytic action a certain, though limited, specificity. Certain protamines for example are not attacked, and it does not hydrolyze the products of its own action on protein. Similarly it appears to exhibit a limited specificity in its synthetic action. Resynthesis is not obtained with all concentrated peptic digests.

The facts that the same number of amino and carboxyl groups are liberated in peptic hydrolysis (1), and that these groups are increased in equal amount also in tryptic and in ereptic hydrolysis, suggest that the specificity exhibited by these hydrolytic enzymes is associated not with the amide linkage *per se*, but with a particular configuration of the molecule attacked.

The possibility that it is the presence or absence of an analogous complex which determines the synthesizeability of a given mixture of protein cleavage products is suggested by the following observations which are described in detail later. It was found that the yield of protein that could be synthesized from a given peptic digest was highest when the hydrolyzing action of the pepsin was stopped as soon as all the protein had disappeared from the solution. The yield grew less the longer the digest was permitted to contain active enzyme after this time. A possible explanation for this progressive diminution in the yield of protein synthesized, with the length of time which is allowed to elapse before the digest is prepared for synthesis, *i.e.* before it is concentrated at pH 4.0 after the pepsin is destroyed by boiling, is that the hypothetical complex essential for peptic synthesis is hydrolyzed in the course of a secondary hydrolysis. It was observed in a systematic investigation of the stages of peptic hydrolysis (2), that a slow, secondary hydrolysis of both proteose and peptone sets in after the first very rapid disintegration of albumin by pepsin.

An attempt was made to ascertain, regardless of the mechanism by which this effect is produced, whether the progressively lessening synthesizeability is due to the action of the pepsin, to the action of the acid, or to their combined action.

A large quantity of a peptic digest of egg albumin at pH 1.6 was prepared After a short interval a fraction was removed and boiled for one half hour in order to destroy the pepsin. Half was then concentrated at pH 4.0; the remainder was allowed to stand at room temperature with its hydrogen ion concentration at pH 1.6, which was the same as that of the main digest, until the last sample was removed from the main digest 23 days later. The reaction was then adjusted to pH 4.0 and this residual fraction was concentrated to the same extent as the first portion which had been prepared for synthesis immediately on removal from the main digest. In this manner a number of fractions were obtained which had been exposed to the combined action of the pepsin and a hydrogen ion concentration of pH 1.6 for varying lengths of time, and a second series in which each member had been exposed to the action of pepsin for the same periods as corresponding members in the first series, but in which the exposure to acid was the same for all as for that of the final sample which had suffered the action of the acid longest, *i.e.* 26 days. All the fractions were concentrated to the same nitrogen content. To 10 cc. of each fraction 1 cc. of a 10 per cent pepsin solution was added, and the mixtures were then set away at 37°C. for two weeks, a period which is adequate for the attainment of equilibrium.

It was found, as before, that the yield of protein synthesized progressively diminished with the length of time which the digest had been exposed to the pepsin; and further that exposure to a hydrogen ion concentration of pH 1.6 in the absence of active pepsin did not affect the amount of the yield. The fractions, in which the reaction was maintained at pH 1.6 for 23 days, 16 days and 9 days after destruction of the pepsin, gave the same yields of plastein as the corresponding fractions which were adjusted to pH 4.0 and concentrated immediately after removal from the main digest. These results indicate that the diminution in synthesizeability of a digest on exposure to pepsin at pH 1.6, is due to the action of the pepsin, and not of the acid, and is presumably a secondary hydrolysis.

It seems improbable that the specificity, *i.e.* the complex essential for peptic synthesis, resides in the amide linkage, in spite of the fact that the product of peptic synthesis contains fewer free amino and free carboxyl groups than any of the constituents of the digest. Were this

specificity associated only with the amide linkage, it would be difficult to account for the failure to effect synthesis in a digest of gelatine, or for the small yields obtainable from a gliadin digest. The problem is the same as in the enzymatic hydrolysis of proteins. Pepsin, trypsin, and erepsin all increase the number of the free amino and free carboxyl groups, yet show a distinct and characteristic specificity in the substrates susceptible to their action.

This effect of the duration of hydrolysis on the yield of protein synthesized precludes, it seems, the possibility that the essential radicle is a single amino acid, or a dipeptide. The latter substances are, as far as is known, not changed by pepsin, and are not, therefore, affected by any length of exposure to the action of that enzyme. Furthermore, the addition of the deficient amino acids to a digest of gelatine, does not remove the inability of such a solution to support peptic synthesis.

It was found that synthesis could also be effected in concentrated solutions of isolated fractions of a peptic digest, *i.e.* of proteose and of peptone. The yields were approximately the same as in similar concentrations of the whole digest, though the proteins so synthesized differed in some respects from those obtained from the whole digest. Assuming the existence of the hypothetical complex suggested above, the synthesizeability of both isolated proteose and peptone would indicate its wide distribution in the protein molecule (or at any rate among its cleavage products).

A series of experiments were carried out which constituted, in a sense, the converse of those, discussed above, in which the synthesizeability of digests hydrolyzed for different periods was measured.

Pepsin was added to a concentrated peptic digest and synthesis was permitted to proceed until no more protein was synthesized. The synthesized protein was removed by filtration after dilution, the filtrate was reconcentrated, its reaction readjusted to pH 4.0 and synthesis again induced with pepsin. This was repeated three times, so that four crops of protein were obtained from one original portion of concentrated digest.

In each case the reaction was allowed to proceed long enough to ensure a maximum yield. The four yields diminished progressively. The cessation of synthesis in any one digest obviously was not due to complete utilization of all the material essential for synthesis. The sim-

plest explanation is that the cessation in each case was due to the attainment of true equilibrium. A previous experiment had led to a similar conclusion. There, artificially synthesized insoluble protein, added to a concentrated solution of digest and active pepsin, was found to inhibit subsequent synthesis to a degree directly proportional to the amount added (3). The result seemed anomalous at that time in that the added protein, a substance apparently not in solution, had influenced the equilibrium position just as if it had been in solution. A similar phenomenon was observed later in the inhibition of hydrolysis by coagulated egg albumin (4).

Recent observations indicate that the synthesized protein is probably soluble to a considerable extent in the concentrated digest, though it is thrown completely out of solution on dilution. When a concentrated digest containing pepsin was poured into a wide, flat dish and left uncovered, the suspension, opaque and creamy in colour on account of insoluble protein, with the evaporation of water, became presently clear and transparent. On the addition of water the opacity reappeared. The same phenomenon was observed during the first few minutes of synthesis in a concentrated solution of proteose. The mixture remained quite clear, yet the occurrence of synthesis was demonstrable by the addition of trichloroacetic acid, by heating quickly to boiling, or by the addition of water. The latter observations on solutions of proteose were suggested by similar observations of Sawjalow (5).

EXPERIMENTAL

The digests employed were peptic digests of egg albumin. Dried egg white (Merck) was dissolved in N/10 HCl and digested with pepsin (Merck) at pH 1.6 and 37°C. for the lengths of time indicated. The hydrolysis was stopped by heating the digest in boiling water for ½ hour. Its reaction was then adjusted to pH 4.0, after which it was filtered and concentrated on a water bath.

Synthesis was carried out as described below for individual experiments. Measured amounts of a solution of pepsin were added to a known volume of digest. The solutions were thoroughly mixed by violent shaking and then incubated at 37°C. Chloroform was added as preservative. At the end of the period indicated the digest was diluted approximately twenty times. The extent of synthesis was estimated by the determination of the total nitrogen before and after precipitation by trichloroacetic acid.

The following experiment shows the progressive diminution in the yield of synthesized protein with the length of time exposed to the hydrolyzing action of the enzyme.

Ten litres of a 5 per cent solution of egg white were digested with pepsin. From time to time 2000 cc. portions were removed and prepared for synthesis as described above. Various lengths of time were allowed to elapse before the destruction of the enzyme and all the digests were concentrated to approximately the same nitrogen content. Two different enzyme concentrations were employed for synthesis. The results in each case were essentially the same as those given in Table I.

TABLE I

The Effect of Duration of Hydrolysis on the Subsequent Yield of Protein Synthesized by Pepsin

Duration of hydrolysis	Duration of synthesis	Total N in 5 cc. conc. digest	N of filtrate in 5 cc. conc. digest	N of synthesized protein in 5 cc. conc. digest
days	*days*	*mg.*	*mg.*	*mg.*
6	3	660	482	178
	11	662	464	198
	15	658	462	196
13	3	682	560	122
	11	674	522	152
	15	676	526	150
21	3	636	548	88
	11	654	522	132
	15	660	524	136
40	3	690	616	74
	11	684	596	88
	15	688	598	90

In order to ascertain whether the lessened synthesizeability on long digestion was due to the action of the pepsin, or of the acid, or of their combined action, 8 litres of 4 per cent egg albumin were digested at 37°C. with 0.4 per cent pepsin at pH 1.6. After 3 days the clear supernatant digest gave no precipitate with 2 per cent trichloroacetic acid. 2 litres were removed and boiled for ½ hour. One litre was then set away at room temperature; the remainder was filtered from the slight flocculant precipitate produced by boiling, and concentrated, after adjustment of its reaction to pH 4.0, so that 550 mg. nitrogen were contained in 10 cc. of solution. A similar procedure was repeated at intervals of 10 days and 17

days after the beginning of digestion. After 26 days the remainder of the digest was boiled and filtered, its reaction adjusted to pH 4.0, and concentrated. The three 1 litre portions in which the pepsin had been previously destroyed at various times, and which had been standing at room temperature since then, were now filtered, their hydrogen ion concentrations adjusted to pH 4.0, and concentrated on water baths.

The final concentrations of the fractions were, as nearly as they could be made, all the same. 1 cc. of 10 per cent pepsin, and 1 cc. of chloroform were added to 10 cc. of each of the digests, the containing vessels were stoppered and set away at 37°C. for 2 weeks. The contents were then analyzed for plastein. The results, given in Table II, show the diminution with prolongation of exposure to active pepsin of the amounts of protein synthesized, and the absence of any significant effect of acid alone.

TABLE II

The Effect of Exposure to Pepsin and to Acid on the Synthesizeability of a Peptic Digest of Egg Albumin

Duration of exposure to pepsin at pH 1.6	Subsequent exposure to a C_H of $10^{-1.6}$	Nitrogen content of 10 cc. of concentrated digest	Synthesis
days	*days*	*mg.*	*per cent of total nitrogen*
3	—	550	42
3	23	570	40
10	—	540	30
10	16	560	29
17	—	550	25
17	9	570	25
26	—	550	20

It had often been observed previously that the highest yields of plastein were obtained when the pepsin was destroyed as soon as all the dissolved protein was digested. The duration of hydrolysis in these cases was not more than 2 days. Under conditions similar to those of Table I the yield was regularly between 230 to 260 mg. N, in 5 cc. of concentrated digest.

The lessened synthesizeability of the digests hydrolyzed for a longer period cannot, it seems, be due to any internal anhydride formation. The ratio of free amino N to total N rose from 23 per cent in the 6 day digest to 27 per cent in the 40 day digest. Internal anhydride formation would have been accompanied by a reduction in this value. The

more rational interpretation is that the radicle essential for synthesis, liberated in the rapid primary hydrolysis, is destroyed in the much slower secondary hydrolysis. The disappearance of this essential compound during the secondary hydrolysis may be taken as evidence that it is more complex than a single amino acid. Since dipeptides are hydrolyzed only by erepsin, it may be assumed that the compound is more complex than a dipeptide.

These results suggest an explanation for the varying synthesizeability of peptic digests of different proteins. Albumin and globulin digests give high yields. Gliadin on the other hand gives low yields and gelatine digests have so far given consistently negative results. The results with the last two proteins can be interpreted in the light of the above results as due either to the failure of appearance of the essential complex hypothecated above during the primary hydrolysis, or to its more rapid destruction. This suggested explanation is in accordance with the variation in internal chemical structure of proteins, which is indicated by the variation in their rates of hydrolysis by enzymes.

Henriques and Gjaldbäk (8) observed that when proteins are partially digested by weak acid or alkali the concentrated digests yield little or no plastein on the addition of pepsin. This observation affords some ground for the belief that the complex essential for peptic synthesis is destroyed by strong acid or alkali, and may help to explain the non-synthesizeability of digests of gelatine because this protein is prepared from collagen by treatment with strong acid.

For the preparation of the proteose and subproteose fractions employed in the experiments discussed above, 9 litres of a peptic digest of albumin were employed. It was boiled for ½ hour and after cooling 360 cc. 10 per cent NaOH were added to bring the reaction to pH 4.0. It was then filtered and from the clear filtrate the proteose was salted out with anhydrous Na_2SO_4 at 33°–36°C. The proteose rose to the surface in a thick, sticky mass. It was skimmed off, redissolved in distilled water and reprecipitated with the same procedure three times. It was found that very little salt had adhered to the proteose, so that no procedures were necessary to remove it as was the case with the subproteose fraction.

The solution from which the proteose was removed was filtered while still warm in order to free it from suspended particles of salted out proteose. The solution was then cooled to about 7°C. to precipitate as much of the hydrated salt as possible. As the anhydrous salt was added and the hydrated form precipitated, a considerable concentration of the solution was attained. To the cold filtrate from

the precipitated salt enough methylated alcohol was added to bring the concentration to 50 per cent. Practically all the salt and none of the nitrogenous constituents are precipitated in this concentration of alcohol. The alcohol was evaporated *in vacuo* at 40°C.

Both the proteose and subproteose fractions, dissolved in water, and free of salt, were concentrated over a water bath to the same nitrogen concentration; they contained 3000 mg. of nitrogen in 100 cc., corresponding to approximately 20 per cent of material.

To 200 cc. of each of these solutions 4 gm. pepsin were added. The peptone solution very soon became opaque. The proteose, however, remained clear for a much longer time, becoming opaque only after a number of hours. This was due not, as it proved, to a slower rate of synthesis in the proteose solution, but to the fact that the protein synthesized in the proteose digest is soluble in the concentrated solution of proteose.

The synthesized protein is slowly denatured, being converted to an insoluble form on standing in the concentrated digest at room temperature. The rate of this denaturation is greater at temperatures above 30°C. It was observed that the protein synthesized from proteose alone was distinctly less soluble in a dilute solution of products than in a more concentrated one. A heavy precipitate of protein is obtained from the concentrated digest by 2 per cent trichloroacetic acid, whereas addition of an equal volume of water induces only a turbidity. If the concentrated solution of proteose containing the protein synthesized from it is brought quickly to the boil, the protein is precipitated as a coagulum.

In the peptone solution the protein appeared to be precipitated as soon as it was formed.

The yields obtained corresponded to those found with similar concentrations of a complete digest. With a concentration of 20 per cent of material in solution the following yields were obtained. Proteose alone yielded 26 per cent, peptone alone 17 per cent, and a mixture of equal parts of similar concentrations of proteose and peptone 22 per cent.

In order to examine the product of synthesis more closely, more proteose and peptone were prepared. Their solutions were concentrated and proteins synthesized from them, with pepsin, as described. The proteins were isolated by dilution of the suspension of synthesized protein and digest to 10 times the volume with distilled water. The precipitated proteins were washed thoroughly, first by

decantation and finally on a filter paper until the wash water gave a negative reaction for chlorides. The proteins were then washed with absolute alcohol and air dried. The free COOH and amino nitrogen in the protein prepared from proteose was measured and also the rate of its hydrolyzeability, and the results compared with those obtained on the substrate from which it was prepared. The free COOH groups were determined by the method of Willstätter (6), and the free amino nitrogen by the micro method of Van Slyke. The results are given in Table III.

TABLE III

A Comparison of the Chemical Characteristics of Proteose with Those of Protein Synthesized from It

	Total N in 10 cc.	Equivalents × 14 of COOH groups in 10 cc. soln.	COOH equiv. × 14 / Total N	Free amino N in 10 cc. solution	Free amino N / Total N
	mg.		*per cent*	*mg.*	*per cent*
Proteose	15.3	3.8	24.9	2.01	13.2
Protein synthesized from proteose	9.2	1.19	12.9	0.99	10.8

TABLE IV

The Action of Acid on Proteins Synthesized from Proteose and Peptone

	Proteose protein		Peptone protein	
	Before soln. in acid	After soln. in acid	Before soln. in acid	After soln. in acid
	mg. N	*mg. N*	*mg. N*	*mg. N*
Free COOH as N/5 in 5 cc.	0.5	1.2	0.5	1.2
Free amino N, mg. in 2 cc.	0.198	0.409	0.39	0.52

Only the rate of its hydrolysis by pepsin could be measured in the protein synthesized from peptone owing to the small amount of material available.

The protein synthesized from proteose is hydrolyzed much more slowly than the plastein synthesized from the whole digest, and similar results were obtained with the protein synthesized from peptone alone.

When dissolved in dilute acid, so that the pH of the solution is in the neighborhood of 1.6 the proteins synthesized from both proteose and

from peptone undergo certain changes which result in the opening up of a considerable number of amide linkages. Large increases in the free amino N and in the free COOH groups occur. These are set out in Table IV.

An analogous phenomenon has been observed by Cohn and Berggren (7) in the effect of alkali on casein. In this case however the increase in COOH groups was exactly equivalent to the increase in NH_2 groups.

Synthesizeability of Residual Digest after Synthesis

300 cc. of a concentrated peptic digest (35 per cent) were set away with 60 cc. of 15 per cent pepsin and 1 cc. of $CHCL_3$ at 37°C. The concentration of pepsin was made so high in order to obtain as large a yield as possible. At the end of 10 days the digest was diluted to 2.5 litres. 3–5 cc. portions were taken for total nitrogen. 40 cc. were pipetted into 10 cc. of 10 per cent trichloroacetic acid and the total nitrogen of the filtrate was determined. The two Kjeldahl determinations gave, after correction for dilution, the amount of protein synthesized. The remainder of the diluted digest was filtered so that all but a trace of the synthesized protein was removed. The filtrate was reconcentrated, after adjustment of the reaction to pH 4.0, until the amount of digest nitrogen, in contradistinction to nitrogen contained in the pepsin added, was the same as in the original concentrated digest before the first synthesis had occurred. More pepsin was added, and the mixture was incubated again at 37°C. At the end of 10 days the digest, now again containing synthesized protein, was again diluted and the procedure described above repeated. This was repeated three times, so that four yields of protein were synthesized from the one digest.

The details are as follows:—

300 cc. digest + 60 cc. 15 per cent pepsin + 1 cc. $CHCl_3$ incubated for 10 days at 37°C. The digest contained 8700 mg. nitrogen in 100 cc. exclusive of nitrogen added in the pepsin. Of this nitrogen 36.3 per cent was synthesized to protein and therefore removed from the solution.

The filtrate from the 2.5 litres of diluted digest was concentrated to 230 cc., 46 cc. of 15 per cent pepsin and 1 cc. chloroform were added, and the digest was again set away at 37°C. The concentration of digest nitrogen was 6400 mg. in 100 cc. although the actual nitrogen content after addition of pepsin was 6540 mg. per 100 cc. After 10 days incubation the digest was diluted to 1850 cc., 3–5 cc. were taken for total N and 40 cc. for precipitation with trichloroacetic acid and determination of the total nitrogen on the filtrate as before. The per cent synthesis here was 11 per cent.

1850 cc. of the second filtrate were concentrated to 170 cc., 40 cc. of 15 per cent pepsin and 1 cc. $CHCl_3$ were added. It was set away again at 37°C. for 10 days. The concentration of digest nitrogen was 6400 mg. per 100 cc. At the end of the 10 day period it was diluted to 1900 cc. The per cent synthesis here was 4 per cent.

1880 cc. were concentrated to 160 cc. 37.5 cc. of 15 per cent pepsin and 1 cc. chloroform were added, and it was set away at 37°C. for 10 days. The per cent synthesis in this 4th crop was 0.7 per cent.

These results show that the cessation of synthesis in any one digest cannot be entirely due to complete utilization of a material essential for synthesis. If that had been the case no synthesis could have been obtained from the first filtrate. The pepsin added does not supply any synthesizeable material. In spite of the fact that commercial pepsin contains proteoses and peptones no synthesis can be obtained from its concentrated solution. Moreover, the very low yield from the third filtrate where there was a considerable amount of pepsin nitrogen, indicates that the enzyme preparation could not have been the source of the synthesizeable material in the first and second filtrates.

SUMMARY

1. Experiments are described in which it was observed that the yield of protein that can be synthesized by pepsin from a given peptic digest is highest when the hydrolyzing action of the pepsin is stopped as soon as all the protein has disappeared from the solution; and that the longer the digest is permitted to contain active enzyme the more the yield diminishes.

2. Exposure of the digest to a hydrogen ion concentration of pH 1.6 in the absence of active enzyme, does not cause a diminution in the amount of protein which can be synthesized from that digest.

3. Synthesis can be effected also in concentrated solutions of isolated fractions of a peptic digest, *i.e.* of proteose and of peptone. The yields are approximately the same as in similar concentrations of the whole digest, though the proteins so synthesized differ in some respects from those obtained from the whole digest.

4. The cessation of synthesis in any one digest is due to the attainment of equilibrium and not to the complete utilization of available synthesizeable material. The amount of the equilibrium yield, on the

other hand, is dependent on the amount of synthesizeable material in the digest.

5. These observations are taken to show that the synthesizeability of a given mixture of protein cleavage products by pepsin depends upon its possession of a special complex in these products. This complex appears as a result of the primary hydrolysis of the protein molecule by pepsin and is decomposed in the slow secondary hydrolysis which ensues as digestion is prolonged.

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