CCLXXIV. STUDIES ON PANCREATIC PROTEINASE. I.

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(Received August 17th, 1935.)

IN the extensive literature concerning the nature and action of trypsin there appears to be no record of a systematic study of factors affecting the earliest stage of protein breakdown by this enzymic complex or by its isolated proteinase component. Waldschmidt-Leitz and his collaborator [1931; 1932; 1933] have shown that the trypsin complex can be separated into several components each of which effects, characteristically, the degradation of substances of more or less definite complexity or structure. Systematic examination of the kinetic behaviour of each enzyme, acting on suitable substrates, should lead to a better understanding of the complex as a whole and should assist in clarifying certain problems concerning which the literature is sometimes inconclusive and contradictory. Estimations of the increasing amino-nitrogen resulting from the action of trypsin on a protein reveal, by themselves, nothing more than the additive effects of the enzymic components acting upon their respective substrates. Such experimental data have only a very limited value in elucidating the nature of the action of any single component.

In the present work attention has been directed toward the first observable stage of protein degradation, namely the process which renders the protein soluble in trichloroacetic acid solution under the conditions defined later. Very little is definitely known as to the nature of this first stage. It probably represents the rupture of a relatively small number of linkages, resulting in the liberation of molecular aggregates essentially similar to the original molecule but of lower molecular weight. Similar conclusions were reached by Bayliss [1907-08], Sherman and Neun [1918], Vahlteich [1929], Blagoveschensky and Sossiedov [1933], Grabar [1933], Sreenivasaya *et al.* [1934]. By definition the change may be regarded as being due to the proteinase. Without attempting to separate the proteinase from the other enzymes of the trypsin complex, studies have been made of the effects of several factors on the initial rate of the first hydrolytic process. By confining attention to this initial constant velocity the influence of the other enzymes can, at this stage, safely be regarded as being practically negligible. This conclusion is supported by experiments such as that summarised in Table I where it is seen that during the first 2.5 min. of hydrolysis 50% of the original protein was rendered soluble in 5% trichloroacetic acid although amino-nitrogen accumulated to the extent of only slightly more than 1% of the amount present after two weeks' action.

The present paper is concerned with the following aspects of the "first-stage" hydrolysis:

- (1) Comparison of the rate of this hydrolysis with that of amino-nitrogen accumulation.
- (2) Comparison of the rates of hydrolysis of various proteins.
- (3) The influence of substrate concentration on the initial velocity of hydrolysis.

- (4) The relation between enzyme concentration and initial velocity.
- (5) The influence of hydrogen ion concentration on the initial velocity.
- (6) The course and order of the reaction.

Materials.

Substrates. The substrate chiefly employed was Kahlbaum's "casein (Hammarsten)" containing 15.4% nitrogen and 0.8% phosphorus. Stock solutions in buffer (p_H 8.7), kept at 8° for 2 to 3 weeks remained perfectly clear and unchanged in the presence of octyl alcohol. Other proteins used were Merck's ovalbumin, haemoglobin, fibrin and a sample of zein prepared from maize.

Buffer. $M/5$ phosphate-borax-sodium hydroxide buffer, p_H 8.7.

Precipitant. To the sample of the digestion mixture was added an equal volume of 10% trichloroacetic acid. This amount was sufficient not only to cause complete precipitation of the proteins present in 1% concentration but also to stop the enzyme action.

Enzyme. Dry fat-free pancreas powder (passed through No. 80 mesh) was prepared by a method essentially similar to that of Willstätter and Waldschmidt-Leitz [1921; 1923]. The solvents used were acetone, acetone-ether and ether. The proteinase activity of the resultant powders remained practically unchanged for at least 20 months when the material was stored in a desiccator at 8–10°.

The enzyme solutions used in all experiments except those which concern the relation between enzyme concentration and reaction velocity were prepared in the following manner. A weighed portion of pancreas powder was triturated in a glass mortar with successive portions of the extracting solution (buffer solution or 50% aqueous glycerol) until a uniform suspension of thin consistency was obtained. This was transferred to a flask and the mortar was washed with extracting solution; for 8 g. powder a total of 100 ml. of solution was used. After the addition of a few drops of octyl alcohol the combined liquids were shaken for 3 hours in a water-bath at 25°; the suspension was then centrifuged at 1200 r.p.m. for 3 hours after which the supernatant fluid was passed through a Seitz filter. A perfectly clear, slightly yellow solution was obtained. When kept at 8° extracts of the powdered gland with phosphate-borax buffer (p_H 8.7) lost no activity in 2 weeks; 1% sodium carbonate extracts lost 54% in 10 days and distilled water extracts lost 63% of their activity in the same time. On the other hand, 50% aqueous glycerol extracts were completely stable for at least 3 months. This advantage is, to some extent, offset by the fact that glycerol appreciably retards the action of the enzyme.

For the investigation of the influence of enzyme concentration on reaction velocity the enzyme solutions were prepared by extracting weighed portions of pancreas powder with 10 ml. buffer solution for 10 minutes, vigorously shaking the suspension. The supernatant fluids obtained after 10 minutes' centrifuging at 1800 r.p.m. were used immediately.

All enzyme solutions were adjusted, when necessary, to the p_H of the buffer before use. It was not found necessary to add any activator such as enterokinase.

Analytical methods.

In view of the objections, previously mentioned, which apply to the use of amino-nitrogen determinations in the solution of the problems under investigation, the rate of disappearance of the protein itself was adopted as the most suitable measure of proteinase activity. For this purpose either of two procedures was employed.

Procedure 1. In this case the entire contents of a digestion tube were treated in obtaining a single reading, several such tubes being used to establish a progress curve. Centrifuge-tubes (50 ml.) were employed, each containing 4 ml. of substrate solution adjusted to the proper p_H and warmed to 25°. At a convenient time, measured on a stop-watch and recorded as zero time, 1 ml. of enzyme solution (25°) was pipetted into the tube, an operation requiring about 2 sec. After the desired time interval 5 ml. of 10% trichloroacetic acid solution were quickly pipetted into the digestion mixture; the precipitate was spun down, washed once with 5% trichloroacetic acid, recentrifuged and dissolved in 3 ml. $N/10$ NaOH; the solution was transferred quantitatively to a 10 ml. volumetric flask and made up to volume. Duplicate samples (1 or 2 ml.) were analysed for nitrogen in a micro-Kjeldahl apparatus of the improved Parnas-Wagner type; approximately $N/125$ H_2SO_4 and $N/125$ NaOH solutions were used for absorption and titration respectively. In order to establish the initial reaction velocity corresponding to a given set of conditions it was customary to employ at least 3, usually 4, such digestion mixtures identical in every respect but incubated for different periods. Usually it was not necessary to allow the most prolonged digestion to proceed for more than 90 sec. A zero-time reading was obtained by allowing the enzyme and precipitant to flow simultaneously into a tube containing substrate or by adding the trichloroacetic acid first and then the enzyme, the precipitate being treated and analysed in the usual way. By plotting hydrolysed protein-nitrogen against time, progress curves were obtained from whose linear portions initial velocities were calculated.

Procedure 2. In this case successive samples were removed from a single digestion mixture contained in an L-tube rocking in a bath at 25°. From the progress curve the initial velocity of hydrolysis was determined. In view of the speed of the reaction it was necessary to observe very carefully certain precautions. The substrate and any necessary buffer solution were first introduced into the tube and allowed to attain the temperature of the bath. A stop-watch was then started and at a convenient point (zero time) the enzyme solution, previously warmed to 25° and contained in a small open glass vessel, was dropped into the tube; the addition and diffusion of the enzyme were complete in about 2 sec. The total volume and p_H were accurately known. Shortly after the addition of the enzyme mixture a 4 ml. sample was quickly pipetted into a 50 ml. centrifuge-tube containing 4 ml. of 10% trichloroacetic acid. The same pipette was used for subsequent samples and was rinsed with digest liquid before use. With practice it was possible to remove the first sample 20 or 30 sec. after the beginning of the reaction and subsequent samples at 20-sec. intervals if desired. Residual protein-nitrogen was determined as previously described.

In both procedures it was necessary to take account of the acid-insoluble nitrogen contained in the enzyme solution. A quantity of the solution equivalent to that used in the digestion was added to a quantity of water equal in volume to that of substrate *plus* buffer; samples were treated with trichloroacetic acid in the usual way and the total nitrogen of the precipitates was determined. The values so obtained were subtracted from the total protein-nitrogen of the digestion samples.

Occasionally, it was desirable, in both procedures, to adopt considerably longer time intervals than those indicated.

Determination of amino-nitrogen. Amino-nitrogen was calculated after titrating the liberated carboxyl groups according to the method of Willstätter and Waldschmidt-Leitz [1921], using $N/50$ alcoholic KOH and thymol blue.

EXPERIMENTS AND DISCUSSION.

Comparison of the rate of the "first-stage" hydrolysis with that of amino-nitrogen accumulation (Table I). Reference to the results has already been made. They show that determinations of amino-nitrogen in the very early stages of hydrolysis do not permit an accurate evaluation of the proteinase activity. Analysis of residual protein, on the other hand, constitutes a reliable index of this activity.

Table I. *Hydrolysis of caseinogen by trypsin; comparison of the rate of the "first-stage" hydrolysis with the rate of amino-nitrogen accumulation.*

Initial caseinogen concentration: 1%.

Enzyme preparation: a phosphate-borax buffer extract.

Residual protein analysed as in procedure 2.

Minutes after zero time	mg. protein-nitrogen hydrolysed	% hydrolysis	mg. amino-nitrogen
0.5	0.54	11.6	0
1.0	1.09	23.3	0
1.5	1.54	33.0	0
2.0	2.12	45.4	0.07
3.0	2.60	55.5	0.16
5.0	3.44	73.5	0.42
10.0	4.06	87.0	0.66
15.0	4.39	94.0	0.70
20.0	4.45	95.0	0.76
30.0	4.68	100.0	0.94
45.0	—	—	1.03
60.0	—	—	1.32
Two weeks	—	—	8.35

Fig. 1. Rates of hydrolysis of caseinogen (1), fibrin (2), haemoglobin (3), zein (4) and ovalbumin (5), by the pancreatic proteinase.

Comparative rates of hydrolysis of different proteins. The rates of hydrolysis of five proteins at 25°, in buffered digests at p_H 8.7 and present, initially, in 0.92% concentration were followed by procedure 2, the same amount of enzyme being used in all cases. The results, expressed in Fig. 1, are of interest for the following

reasons. (1) Of the soluble proteins tested caseinogen was the most rapidly hydrolysed. (2) The order in which the proteins can be arranged on the basis of initial rates of hydrolysis (*e.g.* for 1 min.) is quite different from the order of arrangement based on 5 min. hydrolysis. This difference emphasises the importance of distinguishing, in kinetic experiments, between initial rates and rates calculated from the results of more prolonged digestions. (3) Ovalbumin, known to be very resistant to prolonged tryptic action, was hydrolysed for a short period at a rate comparable with that of the other proteins; subsequently the hydrolysis was very slow. The experiment suggests that an anti-proteinase substance may have been liberated during the very early hydrolysis. In this connection it is of interest to recall that Balls and Swenson [1934] showed that the proteinase inhibitor of egg-white is found in the watery fraction of the white and that this watery fraction is formed during storage of the egg by proteolysis of the thick white.

Relation between substrate concentration and initial velocity of hydrolysis. Northrop's studies on the kinetics of trypsin digestion [1924; 1932-33; Northrop and Kunitz, 1932-33] led him to the view that the enzyme does not form a compound with the substrate and that the speed of the hydrolysis is proportional to the concentration of free enzyme, *i.e.* enzyme not combined with products. This view of the mode of action of trypsin is opposed to the well-known theory of Michaelis and Menten [1913] which is an elaboration of those of Brown [1902] and of Henri [1905]. According to this theory enzyme-substrate compound formation takes place and the speed of the observed reaction depends directly upon the concentration of the compound. The theory was originally founded on experiments with the invertase-sucrose system; in recent years several other enzyme systems have been shown to behave in a manner predictable by the theory.

The investigation of the applicability of the equation of Michaelis and Menten to a given enzyme system is of primary importance in deciding whether or not the enzyme concerned combines with the substrate to yield a compound whose concentration determines the rate of the observed reaction. In such experiments it is not sufficient to calculate the substrate concentration corresponding to half-maximum velocity and to assume that this value represents the dissociation constant of the enzyme-substrate compound. Further analysis of the data is required in order to determine whether combination according to the mass law actually takes place.

A series of caseinogen solutions of different concentrations was prepared from three stock solutions, 5, 1.5 and 0.1%. Two buffer extracts of powdered pancreas were used, the first (Enzyme 1) prepared by extraction with $M/5$ potassium chloride-sodium borate solution p_H 9.3 and the second (Enzyme 2) with $M/5$ phosphate-borate-sodium hydroxide solution p_H 8.7. The final volume of each digest, 5 ml., was adjusted by adding to the substrate solution a suitable amount of the appropriate buffer prior to the addition of the enzyme. The initial rate of disappearance of acid-insoluble protein was followed by procedure 1. Typical results are recorded in Tables II and III, the enzyme preparations being 1 and 2 respectively. The values of K_S and V_{max} in the equation of Michaelis and Menten were obtained by an algebraic method.

The agreement between observed and calculated values is sufficiently close to justify the conclusion that the initial velocity of hydrolysis is predictable by the theory and that caseinogen enters into combination with the proteinase. The difference in the two values of K_S is not easily explained without further investigation, but it may be due to the use of different buffers at slightly different p_H levels. In another experiment in which a 30% glycerol extract of the powder

Table II. *Relation between substrate concentration and initial velocity of hydrolysis.*

Potassium chloride-sodium borate buffer, p_H 9.3.
Temperature 25°.

% caseinogen concentration [S]	Initial velocity	
	Observed (mg. protein-nitrogen hydrolysed per sec.)	Calculated from the equation $v = \frac{0.0434 [S]}{[S] + 0.295}$
0.096	0.0109	0.0107
0.260	0.0200	0.0203
0.432	0.0244	0.0258
0.800	0.0320	0.0317
1.13	0.0352	0.0344
1.78	0.0319	0.0373
2.37	0.0281	0.0386

Table III. *Relation between substrate concentration and initial velocity of hydrolysis.*

Phosphate-borate buffer, p_H 8.7.
Temperature 25°.

% caseinogen concentration [S]	Initial velocity	
	Observed (mg. protein-nitrogen hydrolysed per sec.)	Calculated from the equation $v = \frac{0.1035 [S]}{[S] + 0.234}$
0.050	0.0186	0.0183
0.110	0.0320	0.0332
0.220	0.0485	0.0503
0.380	0.0675	0.0642
0.550	0.0763	0.0730
0.820	0.0790	0.0806
1.08	0.0840	0.0851
1.40	0.0830	0.0887

was used the agreement between calculated and observed values of v was somewhat better than that indicated in Table III, but the value of K_s was considerably lower than the others; the difference was possibly associated in some manner with the known inhibitory effect of glycerol on the activity of the proteinase system. In the range of concentration above 1.5% the observed velocity was always less than the calculated. It is possible that here, as in the case of the invertase-sucrose system [Nelson and Schubert, 1928] the falling off of the observed velocities in the case of the higher substrate concentrations is due to a decrease in the relative water concentration. But, with the proteolytic system, no direct study of this point has been made although experiments on the effects of carbohydrates on proteinase activity, to be described in a later paper, strongly suggest that variations in the relative water concentration seriously affect the activity of the enzyme. The possibility of the combination of the enzyme with two or more molecules of substrate to form an inactive compound [cf. Murray's experiments on liver esterase, 1930] was tested by the method of Lineweaver and Burk [1934]; examination of the available data showed that the falling off was not due to such a combination.

Relation between enzyme concentration and initial velocity of reaction. The results in Table IV show that the initial velocity of hydrolysis (v) varies directly with the enzyme concentration (E). Such a relationship is to be expected on the

basis of the theory of Michaelis and Menten. This finding is in opposition to that recently reported by Bergmann and Pojarlieff [1934] who observed that the initial velocity of collagen breakdown by trypsin was proportional to the square root of E ; a similar observation was made by Bergmann and Föhr [1932] in experiments in which the tryptic digestion of gelatin was followed by an optical method. In both cases, however, consideration of the diffusion factor must affect any generalised interpretation of the results. Bayliss [1925] observed that, depending upon the stage of protein breakdown by trypsin measured by changes in electrical conductivity, the relationship between E and v varied: in the early stages v was directly proportional to E , but in later stages v was proportional to a number of roots of E . Northrop examined the question in some detail: although his data [1924, p. 449] indicate a direct relationship in the early stage at 40° , yet at 0° he found that the Schütz rule was applicable in a rather restricted sense [1924, p. 723]. The complexity of "trypsin" and the variety of reactions catalysed by its various components make it imperative that, in considering the relationship under discussion, attention be confined at any time to only one of the several enzyme systems. Failure to do this leads to much confusion.

Table IV. *Relation between enzyme concentration and initial velocity of hydrolysis.*

Initial concentration of caseinogen 0.80 %.		
p_H 8.6.		
Procedure 1 used.		
Relative enzyme concentration [E]	Observed initial velocity (v) (mg. protein-nitrogen hydrolysed per sec.)	Calculated initial velocity $v = 0.00369 [E]$
1	0.0037	0.00369
3	0.0119	0.0111
8	0.0294	0.0295
12	0.0467	0.0443
16	0.0581	0.0590

Influence of p_H on initial velocity of hydrolysis. The substrates were caseinogen, haemoglobin and fibrin, present in the digestion mixtures in 0.80 %, 0.79 % and 0.55 % concentrations respectively. The enzyme preparation was a phosphate-borax extract and procedure 2 was used. Substrate solutions were prepared by titrating measured amounts of stock solutions to the desired p_H with H_2SO_4 or NaOH solution. The appropriate buffer solutions were then added in sufficient quantity to yield known volumes of substrate solution of the desired concentrations. The initial p_H of each digestion mixture was assumed to be identical with that of a control mixture, determined electrometrically; the controls were in all respects similar to the experimental digests except that boiled enzyme was used in place of the active preparation, the p_H of the boiled and active enzymes having previously been adjusted to the same level. Frequent tests showed that during the short reaction periods adopted (up to 60 sec.) no detectable change in p_H occurred. It was therefore possible to correlate accurately determined values of the initial velocity of hydrolysis with p_H values which were known not to vary throughout the observed course of the reaction. The results are expressed in Fig. 2 where it is seen that all three substrates have the same optimum, p_H 8-9.

In the case of enzymes acting upon substrates which are only very slightly ionised, Euler *et al.* [1924] and Josephson [1925] have shown that the speed of

the reaction is determined by the fraction of the total enzyme existing as undissociated enzyme-substrate compound. This fraction is, in turn, a function of several factors, namely hydrogen ion concentration, acid dissociation constant of the enzyme-substrate compound, substrate concentration and the Michaelis constant. With substrates, such as proteins, which may be very appreciably ionised in solution the equation of Euler *et al.* in its simple form would not necessarily be expected to hold. Northrop [1922-23] observed a close similarity

Fig. 2. Effect of p_H on the initial velocity of hydrolysis of various proteins by the pancreatic proteinase.

between the titration curves of caseinogen, haemoglobin and gelatin on the one hand and, on the other, the p_H -activity curves obtained in experiments on the tryptic hydrolysis of these proteins. From this similarity he concluded that the concentration of protein anions at various p_H levels determined the degree of digestibility of the protein. Trypsin itself he regarded as a strong base behaving as a univalent cation in solutions of p_H 2 to 10, the isoelectric point being at about p_H 10.

Table V.

	Caseinogen	Fibrin	Haemoglobin
Isoelectric point	4.70	5.80*	6.74
p_H corresponding to 50% of maximum activity	6.90	7.20	7.63

* Vonk [1931]—point of minimum swelling in phosphate buffer.

On comparing the isoelectric points of the proteins investigated with the p_H values corresponding to 50% of maximum activity, the relationship in Table V is observed. As the isoelectric point decreases, the lower p_H limit for proteinase activity also decreases. This supports Northrop's conclusion that the enzyme is active only on protein anions. As the p_H approaches 8 the proteinase may begin to lose its positive charge and, in consequence, its effectiveness; but the increased concentration of protein anions balances the loss of activity due to this cause. The hydrogen ion concentration therefore exercises its influence on the proteinase-protein system in two directions: (1) on the ionisation of the protein, (2) on the ionisation of the enzyme or of the enzyme-substrate compound. The identity of the p_H optimum of the three proteins investigated seems to be well established but the limited data do not permit the assumption that other proteins, under the same conditions, would have the same optima.

The course of proteinase action. The application of the expression for a uni-molecular reaction to the course of "trypsin" digestion has frequently been attempted, but usually with very little success. Other equations containing arbitrary constants have also been proposed to express the course of the reaction. In the present work it has been observed repeatedly that the course of the reaction was linear during the breakdown of a considerable portion of the substrate—in some cases, as much as 65 %. Subsequently, the rate of reaction diminished when complicating factors became significant. The true course of the action of the proteinase must therefore be regarded as being of zero order.

Incidentally, the adoption of initial velocity measurements as the basis of kinetic studies provides a method for the comparison of the proteinase activities of different enzyme preparations. Accordingly, the proteinase unit may be defined as the amount of enzyme which causes caseinogen breakdown at an initial rate equivalent to 0.10 mg. nitrogen per sec. under the following standard conditions: total volume of digestion mixture, 5 ml.; caseinogen concentration, 1 %; p_H 8.6–8.7 controlled by phosphate-borax buffer; temperature 25°.

SUMMARY.

1. With the aid of micro-methods, permitting the measurement of the initial velocity of protein hydrolysis, studies of the activity of the pancreatic proteinase have been made. The rate of protein disappearance was much greater than that of amino-nitrogen accumulation. The latter is not a satisfactory index of the activity of the proteinase.

2. The rates of hydrolysis of various proteins were compared.

3. The initial rate of hydrolysis varied with the substrate concentration in a manner predictable by the theory of Michaelis and Menten.

4. The initial rate of hydrolysis varied directly with the concentration of enzyme over a wide range.

5. The influence of p_H on the rate of hydrolysis of three proteins was investigated.

6. The reaction was shown to be one of zero order during the hydrolysis of a considerable fraction of the protein.

7. The results are discussed in relation to those of previous investigators.

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