CRYSTALLINE TRYPSIN

V. Kinetics of the Digestion of Proteins with Crude and Crystalline Trypsin

By JOHN H. NORTHROP

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, June 22, 1932)

The kinetics of trypsin digestion have been studied by several workers (1–8). The results are anomalous from the point of view of the theory of simple catalysis both as regards the effect of the concentration of substrate and the time course of the reaction. These studies have been made with crude pancreatic extracts which undoubtedly contain several enzymes and some of the anomalous results may be due to this fact. The crystalline trypsin prepared by Kunitz and the writer (9) appears to be a chemical individual and certainly contains fewer enzymes than crude pancreas extract. The kinetics of the digestion of gelatin, casein, and hemoglobin with crude pancreatic extract and crystalline trypsin have been studied. The results of these experiments are contained in this paper.

Extent of the Reaction

The crystalline trypsin increases the formol titration of casein by about 100 per cent, equivalent to about 100 hydrolyses per mole, and the formol titration of gelatin solutions by about 200 per cent, equivalent to about 60 hydrolyses per mole. The crude preparations cause about three times as much hydrolysis as does the crystalline (9).

Effect of the Concentration of Substrate

The rate of digestion of various concentrations of gelatin, casein, and hemoglobin with crude or crystalline trypsin were determined at 35°C. The amount of digestion was followed by the increase in formol titration with casein and hemoglobin and also by decrease in

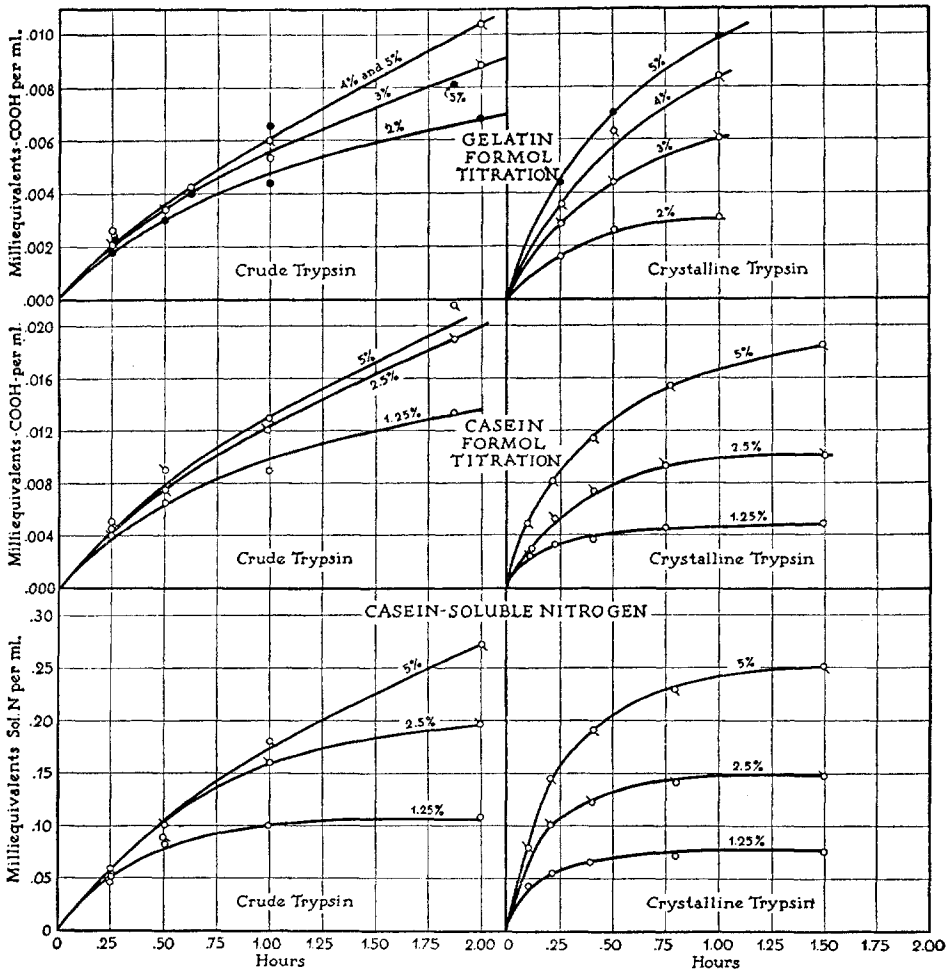

FIG. 1. Digestion of various concentrations of gelatin and casein with crude and crystalline trypsin.

protein soluble in 2.5 per cent trichloracetic acid. The results of these experiments are shown in Figs. 1 and 2. The experiments show that for the first 30–40 per cent of the reaction the amount of digestion with crude trypsin is the same for 2.5 and 5 per cent protein concentration. In other words, the amount of digestion instead of being proportional to the substrate concentration, as predicted by the simple theory, becomes independent of it. This result is frequently observed with enzymes and has usually been ascribed to the formation of an intermediate compound. With the crystalline trypsin this anomalous

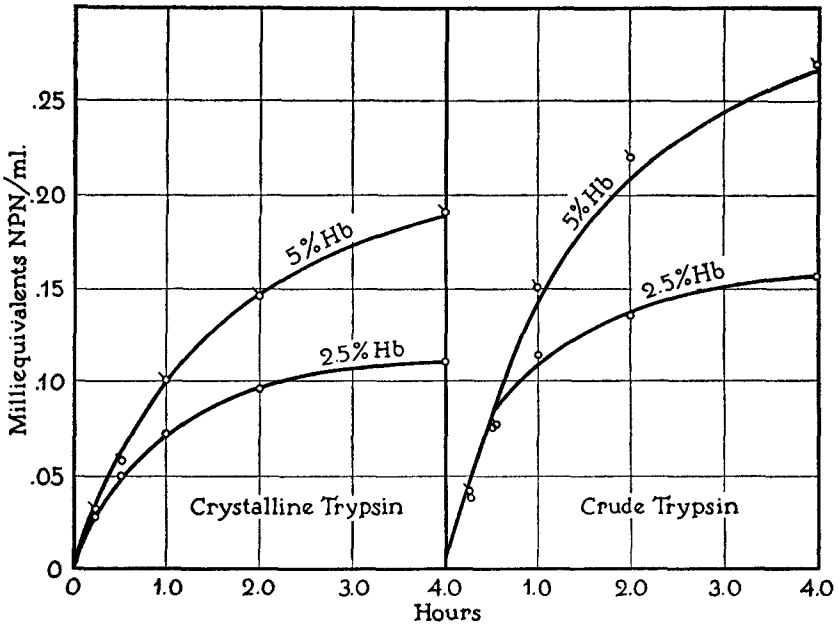

FIG. 2. Digestion of hemoglobin with crude and crystalline trypsin

result is much less marked, the amount of digestion being nearly that expected from the change in the substrate concentration. The first few per cent of the reaction still show this effect, however, especially in the case of casein and hemoglobin when the digestion is followed by means of the change in protein nitrogen.

Kinetics of the Reaction

The digestion of casein as determined by the formation of non-protein nitrogen with the crude trypsin preparation follows the course

of a monomolecular reaction quite closely although the value of the velocity constant is nearly inversely proportional to the protein concentration instead of being independent of this value. This is the result previously obtained (7, 9, 13). With crystalline trypsin the rate of digestion decreases more rapidly than with the crude trypsin and the velocity constants drop quite rapidly as the reaction proceeds. This agrees with the results of Schönfeld-Reiner (11). This is more marked with dilute protein than with concentrated so that with crystalline trypsin the value of the constants for the two protein concentrations approach each other quite rapidly. These results are shown in Table I. The values for the velocity constant have been tabulated at corresponding total amounts of digestion so that they are

TABLE I

Kinetics of Hydrolysis of 2.5 and 5 Per Cent Casein with Crude and Crystalline Trypsin

$K = 1/T \log_{10} A_t/(A_t - A)$

A	Crude trypsin with		Crystalline trypsin with	
	2.5 per cent casein	5 per cent casein	2.5 per cent casein	5 per cent casein
	K	K	K	K
0				
2.0	0.50	0.21	0.084	0.04
4.0	0.54	0.22	0.080	0.04
6.0	0.55	0.22	0.050	0.03
8.0	0.57	0.23	0.030	0.03
10.0	0.50	0.21	0.026	0.026

A_t for 2.5 per cent = 11.0 = final total amount.
for 5.0 per cent = 22.0 = " " "

comparable. This result might be due to the fact that casein is a mixture or solid solution of several different proteins (12). The experiment was therefore repeated with hemoglobin, which is probably a pure substance. The reaction shows the same abnormal behavior with hemoglobin. In this case the monomolecular constants drop quite rapidly with both purified and crude trypsin.

The Rate of Digestion of Mixtures of Casein and Gelatin

The writer has pointed out before (2) that although the effect of changing the substrate concentration agrees with the hypothesis of

intermediate compounds, the results of experiments in which the rate of digestion of a mixture of proteins is followed, do not agree with this hypothesis. If the fact that a 5 per cent gelatin or casein solution digests at the same rate as 2.5 per cent be due to the saturation of the enzyme with the protein, then the addition of casein to a 5 per cent gelatin solution should cause no increase in the rate of digestion since it has already been assumed that the enzyme is saturated with the gelatin. If the rate of digestion of the casein alone is determined in such a mixture, then it would be predicted that the digestion of the casein in the presence of the gelatin would be much slower than the rate of digestion of the same concentration of casein alone. On the other hand, if no intermediate compound is formed then it would be expected that the rate of digestion of a mixture of casein and gelatin would be equal to the sum of the rates of the two proteins separately. In determining the rate, however, it is necessary, owing to the inhibitory effects of the products of the reaction, to compare the curves at a point of equal total digestion rather than at equal time intervals. The quantity of protein digested after a given time interval would not be expected to be equal to the sum of the two quantities separately. This point has been overlooked by Westenbrink (10) who has erroneously quoted the writer as predicting that the amount of digestion of a mixture at a given time would be equal to the sum of the amounts digested in the two solutions separately. Westenbrink's results even when recalculated, however, show less difference between the rate of digestion of mixtures of casein and gelatin and of gelatin alone than the writer's experiments, so that the experiments have been repeated. The results of experiments in which the digestion of mixtures of 5 per cent casein and 5 per cent gelatin, are compared to the rates of digestion of the two proteins separately, are shown in Fig. 3. They confirm the earlier experiments of the writer (2) in that the rate of digestion of the mixture is greater than the rate of digestion of either protein alone and is nearly equal to the sums of the rates of the two proteins alone, especially in the case of the crystalline trypsin. The lower part of Fig. 3 shows that 5 per cent casein in the presence of 5 per cent gelatin digests at identically the same rate as does 5 per cent casein alone. As stated before (3), this result is difficult to account for on the basis of an intermediate compound unless it be further assumed that there are two enzymes, one for each protein. It cannot

be assumed that the casein alone digests in the mixture since the rate of hydrolysis of the casein is the same in the mixture as in the pure casein while the increase in formol titration is much greater in the

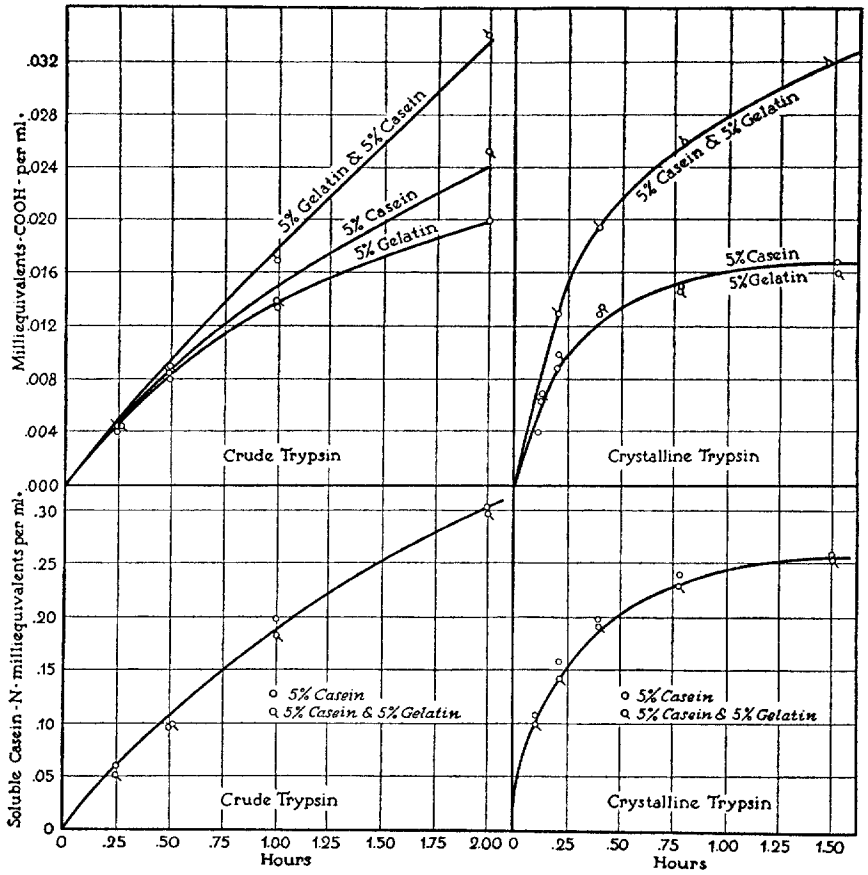

FIG. 3. Digestion of mixtures of casein and gelatin with crude and crystalline trypsin. Upper figures show increase in formol titration of mixture and of two proteins separately and lower figures show digestion of casein when present alone and in presence of gelatin.

mixture than in the casein solutions alone; also, the viscosity of the gelatin-casein mixture drops rapidly. These results show that the gelatin as well as the casein is hydrolyzed in the mixture.

Kinetics of the Reaction as Determined by Changes in Viscosity

The preceding experiments show that the abnormalities of the reaction between trypsin and proteins are less marked when purified trypsin solutions are used. The purified trypsin preparations contain a smaller number of enzymes than the crude preparations and the reaction does not proceed nearly so far with the purified as with the crude enzyme (9). These results indicate that the abnormal character of the reaction is due to the presence of a series of consecutive reactions rather than to the formation of an intermediate enzyme substrate compound and this conclusion is strengthened by the results of the experiments on mixtures of proteins. If this explanation is correct it would be expected that the results would agree better with those predicted from the simple theory of catalysis if the reaction were followed by a method which determined only the first step in the digestion and which was not affected by the subsequent reactions. The change in viscosity of the protein solutions offers such a method since the protein molecule itself is almost entirely responsible for the high viscosity and the viscosity is changed very slightly, if at all, by subsequent further hydrolysis. The method has the disadvantage that the physical significance of the change in viscosity is somewhat uncertain. The value for the viscosity itself cannot be used since this does not increase in proportion to the protein concentration and has no simple physical significance. All theories of viscosity agree that the viscosity is some function of the volume occupied by the solute, and Kunitz (14) has found an empirical equation which gives very reasonable values for the volume of the solute calculated from the viscosity. In calculating the results of the viscosity measurements, therefore, the values for the volume of the solute occupied by the hydrated protein molecules have been interpolated from the observed viscosity of Kunitz's equation. These values have then been corrected for the final volume occupied by the solute at the end of the reaction. The results of an experiment calculated in this way for the hydrolysis of casein with purified trypsin are shown in Table II. The values under K are those for the monomolecular equation using common logarithms and time in hours. They are reasonably constant and furthermore the value of the constant is about the same for 3 per cent and 5 per cent casein; *i.e.*, the reaction

TABLE II

Kinetics of Digestion of Various Concentrations of Casein with Crystalline Trypsin as Determined by Changes in Viscosity

$$K = \frac{1}{T \text{ hrs.}} \log_{10} \frac{V_o - V_e}{V_t - V_e}$$

Time	5 per cent casein				3 per cent casein			
	η_S	V	$V-V_e$	K	η_S	V	$V-V_e$	K
hrs.								
0	2.30	17.3	9.4		1.57	9.5	4.4	
0.16	1.975	14.3	6.4	1.00	1.463	8.1	3.0	1.0
0.22	1.905	13.6	5.7	1.00	1.424	7.7	2.6	1.0
0.63	1.686	11.1	3.2	0.80	1.34	6.4	1.3	0.85
1.71	1.532	9.2	1.3	0.75	1.288	5.5	0.4	0.62
25.00	1.439	7.9			1.282	5.4		

TABLE III

Kinetics of the Hydrolysis of Various Concentrations of Gelatin with Crude and Purified Trypsin as Determined by Changes in Viscosity

$$K = \frac{1}{T \text{ hrs.}} \log_{10} \frac{V_o - V_e}{V_t - V_e}$$

Concentration gelatin, *per cent*		5				3				1			
	Time	η_S	V	$V-V_e$	K	η_S	V	$V-V_e$	K	η_S	V	$V-V_e$	K
	hrs.												
Crystalline trypsin	0	4.13	27.5	20.7		2.31	17.4	12.9		1.285	5.4	3.6	
	0.20	3.10	22.5	14.7	0.70	1.94	14.0	9.5	0.65	1.23	4.5	2.7	0.63
	0.43	2.55	19.0	12.2	0.54	1.67	10.9	6.4	0.70	1.17	3.7	1.9	0.64
	0.67	2.25	16.8	10.0	0.48	1.55	9.3	4.8	0.64	1.15	3.1	1.3	0.66
	1.18	1.98	14.4	7.6	0.40	1.43	7.7	3.2	0.52	1.12	2.8	0.8	0.50
	2.66	1.68	11.1	4.3	0.26	1.33	6.0	1.5	0.69	1.115			
	48.00	1.37	6.8	0		1.23	4.5			1.092	1.8		
Crude trypsin	0	3.75	26.0	19.0		2.19	16.3	11.7		1.26	5.1	3.3	
	0.20	3.19	23.1	16.1	0.36	1.975	14.3	9.7	0.40	1.218	4.5	2.7	0.43
	0.95	2.50	18.8	11.8	0.22	1.69	11.2	6.6	0.25	1.16	3.5	1.7	0.30
	2.00	2.15	16.0	9.0	0.17	1.54	9.3	4.7	0.20	1.137	3.0	1.2	0.22
	4.00	1.91	13.6	6.6	0.11	1.44	7.8	3.2	0.15	1.115	2.5	0.7	0.16
	48.00	1.40	7.0	0		1.24	4.6			1.09	1.8		

is normal or nearly so when followed in this way. The results of an experiment with 1, 3, and 5 per cent gelatin digested with crystalline

and crude trypsin have been calculated in the same way (Table III). In this case, also, the value for the monomolecular constant is independent of the protein concentration as would be expected for a simple monomolecular reaction and in the case of crystalline trypsin the constant decreases only slightly during the first 50 per cent of the reaction. With crude trypsin the constant decreases more rapidly during the course of the reaction but still has about the same value for the different concentrations of protein. These experiments were carried out with about the same concentration of enzyme as were the preceding ones so that the difference is not due to differences in enzyme concentration.

Experimental Procedure

Protein Solutions.—Isoelectric gelatin was prepared as described by Northrop and Kunitz (15). The casein used was Kahlbaum "casein according to Hammarsten." 25 gm. of the protein were dissolved in 400 ml. of M/10 pH 7.6 phosphate buffer and the resulting solution titrated with alkali to pH 7.6 and made up to 500 ml. with M/10 pH 7.6 phosphate buffer. Solutions of lower concentrations were made by dilution of this 5 per cent solution with M/10 pH 7.6 phosphate buffer so that the solutions used were at pH 7.6 and contained M/10 phosphate buffer.

Preparation of Hemoglobin Solution.—Crystalline hemoglobin solution dialyzed free from sulfate at the isoelectric point, titrated to pH 8.6, and diluted to 5 per cent hemoglobin concentration. Total alkali concentration about 0.02 normal. The flask containing the solution was immersed in boiling water for ½ hour to denature the hemoglobin since trypsin does not attack native hemoglobin. 2.5 per cent hemoglobin solution prepared from this solution by dilution with water.

The trypsin preparations were prepared from pancreatic pressed juice as described in a previous paper (9).

The formol titration was determined on 2 ml. of the protein solutions with N/50 sodium hydroxide as described previously (16).

Determination of Soluble Protein Nitrogen.—1 ml. of the digestion mixture was added to 10 ml. of 2.5 per cent trichloracetic acid; the suspension warmed to 70°C. for 10 minutes, cooled to 20°C. for ½ hour, and centrifuged. The precipitate was washed once with 10 ml. of 2.5 per cent trichloracetic acid, centrifuged again and the precipitate dissolved in 2 ml. of N/10 sodium hydroxide, and reprecipitated by the addition of 10 ml. 2.5 per cent trichloracetic acid. The suspension was centrifuged, the precipitate dissolved in 1 to 2 ml. of N/10 sodium hydroxide, and the solution made up to 10 ml. The nitrogen in 5 ml. of this solution was then determined by micro Kjeldahl. This gives the amount of insoluble casein nitrogen. The quantity of soluble nitrogen is then found by subtracting the figure for the soluble nitrogen from the total amount of nitrogen present at the beginning of the reaction. All reactions were carried out at 35°C.

SUMMARY

The rate of digestion, as determined by the increase in non-protein nitrogen or formol titration, of casein, gelatin, and hemoglobin with crystalline trypsin preparations increases nearly in proportion to the concentration of protein, but with crude pancreatic extract the rate of digestion becomes independent of the protein concentration in concentrations of more than 2.5 per cent. With both enzymes the rate of digestion of mixtures of 5 per cent casein and gelatin is greater than would be expected from the point of view of a compound between enzyme and substrate. The rate of digestion of 5 per cent casein in the presence of 5 per cent gelatin is exactly the same as that of 5 per cent casein alone. This result is obtained with both enzymes. The digestion of casein with crude trypsin follows the course of a monomolecular reaction quite closely while with purified trypsin the velocity constant decreases as the reaction proceeds. In the case of hemoglobin the monomolecular velocity constant decreases with both purified and crude enzyme.

When the reaction is followed by changes in the viscosity of the solution the abnormal effect of changing substrate concentration disappears and the reaction is in fair agreement with the monomolecular equation. The results as a whole indicate that the abnormalities of the reaction are due to the occurrence of several consecutive reactions rather than to the formation of a substrate enzyme compound.

REFERENCES

1. Bayliss, W. M., *Arch. sc. biol.*, 1904, **11,** suppl., 261.
2. Northrop, J. H., *J. Gen. Physiol.*, 1922, **4,** 487.
3. Northrop, J. H., *J. Gen. Physiol.*, 1924, **6,** 239.
4. Northrop, J. H., *J. Gen. Physiol.*, 1924, **6,** 439.
5. Northrop, J. H., *J. Gen. Physiol.*, 1924, **6,** 723.
7. Northrop, J. H., *J. Gen. Physiol.*, 1924, **6,** 417.
8. Taylor, A. C., *Univ. California Pub. Path.*, 1907, **1.**
9. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932, **16,** 295.
10. Westenbrink, H. G. K., *Arch. nêerl. physiol.*, 1931, **16,** 528.
11. Schönfeld-Reiner, R., *Fermentforschung*, 1930, **12,** 67.
12. Linderström-Lang, K., *Z. physiol. Chem.*, 1928, **176,** 76.
13. Merrill, H. B., *J. Gen. Physiol.*, 1926, **10,** 217.
14. Kunitz, M., *J. Gen. Physiol.*, 1926, **9,** 715.
15. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1927–28, **11,** 477.
16. Northrop, J. H., *J. Gen. Physiol.*, 1926, **9,** 767.