[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

On the Formation of Chymotrypsin from Chymotrypsinogen

BY J. A. V. BUTLER[1]

The conversion of chymotrypsinogen into chymotrypsin by trypsin was discovered by Kunitz and Northrop[2] and shown to be a simple unimolecular catalytic reaction. In connection with the more extended study of the kinetics of trypsin action reported in the following paper, the activation energy of this reaction was required. Determinations of the velocity constant have been made at several temperatures (see Table I and Fig. 1), from which it is found that the activation energy has the comparatively high value of 16,300 calories. The reaction is abnormally rapid for this energy and the significance of this fact is discussed in the following paper.

TABLE I

VELOCITY CONSTANTS OF CONVERSION AT VARIOUS TEMPERATURES

Reaction mixture: 7 ml. M/15 phosphate buffer, *p*H 7.5; 1 ml. dialyzed chymotrypsinogen solution (total nitrogen = 1.26 mg. per ml.); 1 ml. trypsin solution containing 0.0111 mg. protein nitrogen per ml. Hemoglobin activity = 0.00118 $[T.U.]^{Hb}_{ml.}$; taking specific activity of pure trypsin as 0.16 $[T.U.]^{Hb}_{mg.\ N}$ the purity of trypsin was 0.67%. Concentration in solution added is 0.046 mg. trypsin per ml., or taking the molecular weight of trypsin as 36,500 = 1.27×10^{-9} mole per ml. The concentration of trypsin in the reaction mixture was 1.4×10^{-7} mole per liter.[a]

	0°C.	6.3°C.	13.0°C.	19.6°C.
k min.$^{-1}$	3.35×10^{-3}	5.7×10^{-3}	13.4×10^{-3}	24.3×10^{-3}
k sec.$^{-1}$ for 1 mole trypsin per liter	3.97×10^{2}	6.8×10^{2}	15.9×10^{2}	29.0×10^{2}

[a] I am greatly indebted to Dr. Margaret R. McDonald for carrying out the trypsin assay quoted here.

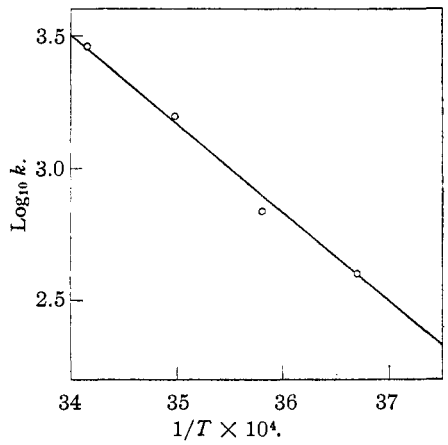

Fig. 1.—Action of trypsin on chymotrypsinogen (log k against $1/T$).

(1) Fellow of the Rockefeller Foundation.

(2) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **18**, 433 (1935).

Some experiments designed to elucidate the nature of the reaction also were carried out. There is an increase in the formol titration in the course of the reaction (Fig. 2) which parallels the growth of enzymatic activity at the beginning but continues to increase slowly when the conversion is completed. It follows that the conversion is accompanied by a small amount of some secondary action, as has also been found in other experiments.[2] The primary increase of the formol titration is most simply interpreted as being due to the liberation of acid groups produced by the splitting of peptide bonds.

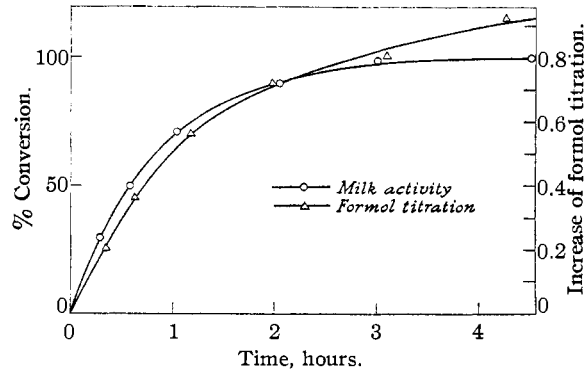

Fig. 2.—Increase of formol titration during activation. Composition of reaction mixture: 6 ml. chymotrypsinogen (9.1 mg. protein N/ml.); 1 ml. *p*H 7 buffer; 0.5 ml. trypsin solution (0.1 mg. N/ml.); temperature, 13.5°.

The number of peptide bonds split can be estimated in two ways. (1) The increase of formol titration during the activation amounts to about 7.5×10^{-6} equivalent in a solution containing 1.3×10^{-6} g. mole per ml. of chymotrypsinogen, or about 6 equivalents per g. mole, *i. e.*, the conversion produced 6 acid groups in the molecule. (2) The initial rate of hydrolysis as measured by the formol titration can be compared with the rate of formation of chymotrypsin molecules. The velocity constant of the reaction was 2.0×10^{-2} min.$^{-1}$, *i. e.*, at the outset $2.0 \times 10^{-2} \times 1.3 \times 10^{-6} = 2.6 \times 10^{-8}$ g. mole per ml. of chymotrypsinogen is converted initially per minute, while the initial rate of increase of the formol titration is 10^{-7} equivalent per minute. The conversion of each molecule thus involves 4 equivalents. The two methods therefore lead to the

conclusion that, in the formation of a chymotrypsin molecule, from 4 to 6 equivalents of acid are liberated.

There is, however, no evidence that any appreciable quantity of nitrogenous material is split from the chymotrypsinogen molecule in the course of the reaction. Kunitz and Northrop[2] found that a slow increase in the amount of non-protein nitrogen accompanies the reaction and continues when the conversion is completed. They regarded this as being mainly formed by a side reaction, leaving open the question whether any part of it was directly associated with the formation of chymotrypsin. This experiment was repeated using a much higher concentration of trypsin, so that the conversion was completed in less than an hour (Table IV). The increase in the amount of "non-protein" nitrogen during the conversion was certainly less than 1% of the total nitrogen present, the accuracy of the determination being of the order of 0.5%. The increase to be expected, if each peptide bond broken releases only 1 nitrogen atom, is from 1 to 1.5%. It is therefore probable that no molecules containing nitrogen are split off.

Since trypsin hydrolyzes the terminal amide group of benzoyl-arginine amide,[3] it appeared to be possible that its action causes the hydrolysis of one or more amide groups. It was found that the amount of free ammonia liberated in the conversion was negligible (Table V).

TABLE II

RATE OF REACTION IN PRESENCE OF DEUTERIUM OXIDE

Reaction mixture: 8 ml. water or 70 per cent. deuterium oxide; 0.5 ml. of chymotrypsinogen solution, 0.5 ml. 1 M pH 7.5 phosphate buffer, 0.2 ml. trypsin (0.05 mg. nitrogen per ml.)

Time, minutes	30	60	90	120	160
Per cent. conversion water	30.5	68	76	82	85
Per cent. conversion deuterium oxide	41.5	69	74	78	83

TABLE III

EFFECT OF CONCENTRATION OF CHYMOTRYPSINOGEN ON VELOCITY CONSTANT

Reaction mixture: 4.5 cc. chymotrypsinogen solution in M/10 *p*H 7.5 buffer, 0.5 cc. trypsin (approximately 0.01 mg. nitrogen per ml.) 25°

c, mole per liter	1.32×10^{-3}	0.15×10^{-3}	0.015×10^{-3}
k, hours^{-1}	0.42	0.58	0.58

TABLE IV

FORMATION OF NON-PROTEIN NITROGEN IN CONVERSION OF CHYMOTRYPSINOGEN TO CHYMOTRYPSIN

Reaction mixture: 10 ml. dialyzed chymotrypsinogen solution containing 3.68 mg. nitrogen per cc.; 0.5 ml. trypsin, approximately 1 mg. nitrogen per cc.

Time, hours	Conversion, %	Non-protein nitrogen, %
0	0	3.9
1	80	4.5
2	90	3.7
23	100	6.3
47	...	10.6
71	...	10.4

TABLE V

LIBERATION OF FREE AMMONIA IN FORMATION OF CHYMOTRYPSIN

Reaction mixture: 30 ml. dialyzed chymotrypsinogen solution containing 10 mg. nitrogen per ml. at *p*H 7.5, 1 ml. trypsin, 0.1 mg. nitrogen per ml. The activation was practically complete in 0.5 hour.

Time, hours	Free NH_3 distilled from 5 ml. as mg. nitrogen
0	0.016
0.5	.026
18	.020
66	.030

TABLE VI

EFFECT OF SALT CONCENTRATION ON THE REACTION VELOCITY

Composition of reaction mixture: chymotrypsinogen solution 1 ml., M/2 *p*H 7.5 phosphate solution 1 ml., salt solution of appropriate strength 6 ml., trypsin (0.01 mg. nitrogen per ml.) 1 ml.

Salt concn., N	Relative velocity constants KCl	NaCl	Na_2SO_4
0	1.0	1.0	1.0
0.5	0.25	0.50	0.63
1.0	.10	0.26	.53
1.5	.08	..	.44
2.0	..	..	.54

It is possible, of course, that an acid containing no nitrogen is liberated, but no acids of this kind have been detected in proteins, and it is much more probable that the reaction is the opening of peptide bonds in closed rings. Very little information about the mode of action of proteolytic enzymes has been obtained. The fact that the rate of the activation is completely uninfluenced when the hydrogen in the solvent is largely replaced by deuterium (Table II) shows that the reaction is not similar to a hydrogen ion hydrolysis and does not involve protons in any direct way. This is in contrast to the behavior of emulsin on glucosides.[4]

Kunitz and Northrop found that the velocity constant of the reaction was independent of the chymotrypsinogen concentration up to 0.42 mg. of protein nitrogen per ml. (8×10^{-5} mole per liter). To find whether a stable complex exists

(3) M. Bergmann, J. S. Fruton and H. Pollok, *J. Biol. Chem.*, **127**, 643 (1939).

(4) K. F. Bonhoeffer, *Erg. der Enzymforschung*, **6**, 47 (1937).

between the enzyme and the substrate, determinations were made at still higher concentrations and it was found (Table III) that the velocity constant was only 20% smaller at a chymotrypsinogen concentration of 1.3×10^{-3} mole per liter than at low concentrations. This diminution might be due to secondary effects arising from the increased concentration rather than approaching saturation of a complex; but it is clear that the Michaelis constant is greater than 1.3×10^{-3} mole per liter.

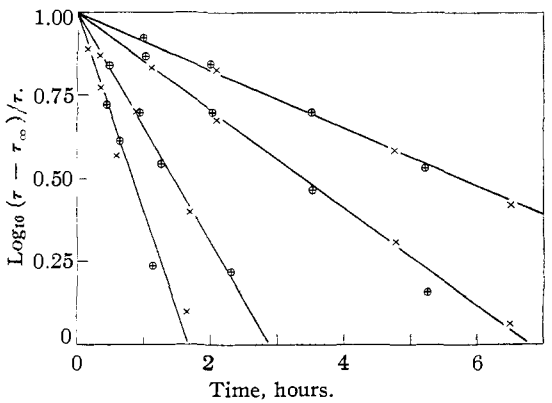

Fig. 3.—Conversion of chymotrypsinogen into chymotrypsin at various temperatures (two distinct experiments at each temperature).

Some experiments were made (Table VI) on the effect of salts on the rate of the reaction.[5] All the salts tried depress the rate, the effect being in the order KCl > NaCl > Na_2SO_4 for equal normalities. Up to 1 *N*, the logarithm of the rate varies roughly linearly with the salt concentration, but at higher concentrations the change is smaller and with sodium sulfate the rate reaches a minimum and begins to rise again. This effect is probably produced by the salting-out action of the salt on the reaction complex. If the reaction complex between the chymotrypsinogen and the trypsin is salted-out to a greater extent than the two constituents, this behavior would occur. The reaction complex is probably negatively charged since the experiments were made at a *p*H at which chymotrypsinogen is negatively charged, while trypsin is in the vicinity of its isoelectric point. The marked difference between potassium and sodium salts indicates a marked cation effect, which might be expected for a negative complex.

(5) *Cf.* M. R. McDonald and M. Kunitz, *J. Gen. Physiol.*, **25**, 53 (1941).

Experimental

Determination of Rate of Activation.—The amount of chymotrypsin formed was determined by its milk clotting action using Herriott's method of observing the clotting time.[6] The concentration of chymotrypsin is taken as inversely proportional to the clotting time τ. If τ_∞ is the final clotting time, the fraction of chymotrypsinogen converted at any time is τ_∞/τ. The velocity constant was obtained by plotting log $(\tau_\infty - \tau)/\tau$ against time (Fig. 3). The two runs which were carried out at each temperature were in good agreement with each other and gave close to a linear relation in this plot.

Determination of Non-protein Nitrogen.—One ml. of a suitable dilution is added rapidly to 10 ml. of 5% trichloroacetic acid at 95–100° and the mixture cooled in a water-bath at 25° and filtered. The nitrogen present in an aliquot part of the filtrate was determined by a semimicro Kjeldahl method.

Determination of Free Ammonia.—Basic phosphate is added to 5 ml. of the solution until just pink to phenolphthalein and then 5 ml. of saturated borax is added.[7] The solution is distilled for fifteen minutes under reduced pressure at 40–42° into boric acid containing 0.1% brom cresol green. This was titrated back to a standard color by N/70 hydrochloric acid.

Formol Titration.—To 1 ml. of the solution add 0.5 ml. of 40% formaldehyde and titrate with 0.01 N sodium hydroxide to the first definite pink color. In cases where the "initial" titer is large, 1 ml. of a more concentrated alkali, which brings the initial solution into the vicinity of the end-point, is added to each sample.

Summary

1. From the velocity constants of the conversion of chymotrypsinogen into chymotrypsin by trypsin at four temperatures, the activation energy of the reaction is found to be 16,300 calories.

2. The increase in the formol titration in the reaction corresponds to a splitting of 4 to 6 peptide bonds. Since very little non-protein nitrogenous material and practically no ammonia are liberated in the reaction, it is probable that the reaction is the opening of peptide bonds in ring structures.

3. The effects of some inorganic salts, of substituting deuterium oxide for water in the solvent, and of chymotrypsinogen concentration on the reaction rate have been observed.

PRINCETON, N. J. RECEIVED AUGUST 5, 1941

(6) R. M. Herriott, *ibid.*, **21**, 501 (1938).

(7) G. Pucher, H. B. Vickery and C. S. Leavenworth, *Ind. Eng. Chem., Anal. Ed.*, **7**, 152 (1935).