Kinetics of the Tryptic Hydrolysis of the Oxidized B Chain of Bovine Insulin*

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ABSTRACT: A quantitative study has been made of the rate of hydrolysis of the two bonds (arginyl$_{22}$ and lysyl$_{29}$) in the oxidized B chain of insulin which are susceptible to the action of trypsin. The analytical data were obtained from a chromatographic system which made use of the amino acid analyzer. The reaction follows two different pathways, each with different intermediates, to yield the same products. Values for the four kinetic constants which describe the over-all reaction were obtained from the composition of the reaction mixture as a function of time. Calculations were facilitated by the use of a FORTRAN program written for an IBM 7040/7094 computer. Trypsin hydrolyzed the arginyl bond 25 times faster than the lysyl bond of the oxidized B chain at pH 8.0 and 30°. The difference in lability of these bonds can probably be attributed to differences in the adjacent amino acid residues rather than to differences in accessibility of the groups to the enzyme owing to secondary or tertiary structure, the C-terminal Pro-Lys-Ala-OH sequence being more resistant to hydrolysis than the internally located Glu-Arg-Gly sequence. Optical rotatory dispersion measurements indicated that the oxidized B chain existed largely as the random coil.

The difference in relative rates of hydrolysis became more pronounced when the pH was elevated (35:1 at pH 10.0 and 52:1 at pH 10.7; at 30°) or when the temperature was reduced (30:1 at 10° and pH 9.0). Since in going from pH 9.0 to 10.7, the ϵ-NH_2 group of lysine is largely discharged while the guanidinium group of arginine is not affected, the differing effect of pH on the rate of hydrolysis of the arginyl *vs.* lysyl bond is attributed to differences in the strength of the side-chain basic groups. Preliminary experiments with the effect of high pH on the tryptic hydrolysis of the model peptides, benzoylarginamide and benzoyllysinamide, support this conclusion.

The action of trypsin on bovine insulin has been the subject of several investigations (Harris and Li, 1952; Nicol, 1960; Laskowski *et al.*, 1960; Young and Carpenter, 1961). The enzyme catalyzes the hydrolysis of only two bonds, both of which are located on the B chain of insulin (the bonds between Arg$_{22}$-Gly$_{23}$ and Lys$_{29}$-Ala$_{30}$). Complete action of the enzyme results in the formation of alanine, a heptapeptide (Gly-Phe-Phe-Tyr-Thr-Pro-Lys) and a tryptic-resistant core, desoctapeptide insulin. Carpenter and Baum (1962) noted that under certain conditions (but not all), alanine and the heptapeptide appeared at the same rate upon treatment of intact insulin with trypsin. More recently, studies have been performed on the oxidized B chain of insulin (Keil and Keilova, 1964; Keilova and Keil, 1964; Wang and Carpenter, 1965). Although the data did not allow the calculation of kinetic constants, it was apparent that the arginyl bond was hydrolyzed much faster than the lysyl bond in this fragment of the insulin molecule. The present investigation was instigated in order to obtain quantitative data on the relative rates of the tryptic hydrolysis of these two bonds in the oxidized B chain of insulin and to study the effect of pH and temperature on these rates. In this connection, it should be noted that Hofmann and Bergmann (1941) have compared the relative rates of hydrolysis of arginyl and lysyl bonds in several model peptides (N^{α}-benzoyl-L-arginamide *vs.* N^{α}-benzoyl-L-lysinamide and benzoylglycyl-L-arginamide *vs.* benzoylglycyl-L-lysinamide) (Bergmann, 1942). In these model peptides, the first-order rate constants, *at pH 7.5*, for the tryptic hydrolysis of the arginine derivatives were about twice as large as those of lysine derivatives. In order to make a comparison between the effect of pH on the hydrolysis of model compounds and the oxidized B chain, we are also reporting preliminary experiments on the effect of pH upon the initial velocities for the tryptic hydrolysis of N^{α}-benzoyl-L-arginamide (BAA)[1] and N^{α}-benzoyl-L-lysinamide

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[1] BAEE, BAA, and BLA refer to N^{α}-benzoyl-L-arginine ethyl ester, N^{α}-benzoyl-L-arginamide, and N^{α}-benzoyl-L-lysinamide, respectively. B is the oxidized B chain of bovine insulin, B-1 is desalanine B chain, B-8 is desoctapeptide B chain, Oct is the octapeptide (Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala), and Hept is the heptapeptide (Gly-Phe-Phe-Tyr-Thr-Pro-Lys). TPCK-trypsin refers to trypsin which has been treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

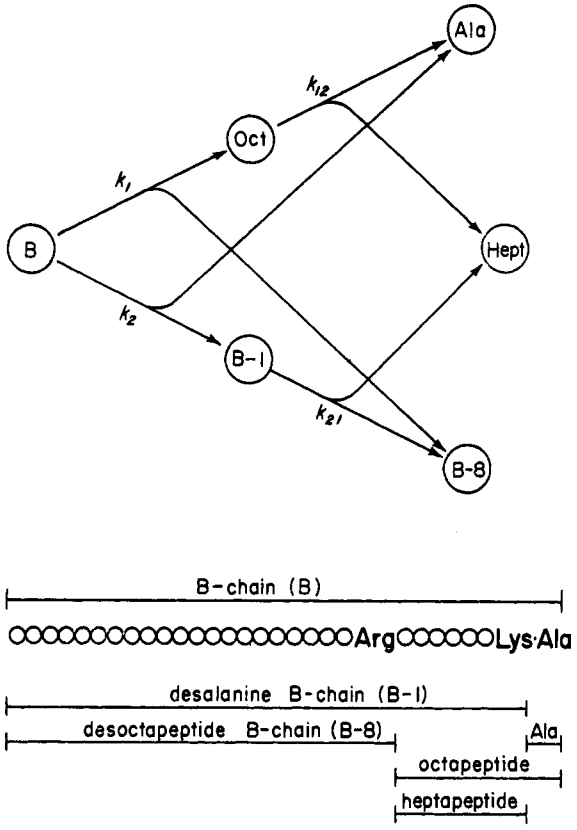

FIGURE 1: A scheme for the action of trypsin on oxidized B chain of bovine insulin.

(BLA).

The tryptic hydrolysis of the oxidized B chain of insulin consists of a reaction with a dual pathway. Each pathway contains two consecutive reactions yielding the same final products (Figure 1). This system represents one of the simplest examples for reaction between endohydrases and their polymer substrates in that only two susceptible bonds are involved. Reactions of the general type depicted in Figure 1 are frequently encountered. To mention but a few recent examples, the hydrolysis of tripeptides (Long and Truscott, 1963), the hydrolysis of prephenic acid diethyl acetal (Plieninger *et al.*, 1965), and the substitution of a bifunctional molecule as illustrated by the formation of trimethylsilyl ethers of bile acid methyl esters (Briggs and Lipsky, 1965) are dual pathway reactions similar to that depicted in Figure 1. With the advent of rapid and precise analytical techniques for complex mixtures, such as automatic ion-exchange and gas–liquid partition chromatography systems, a number of these reactions are amenable to kinetic analysis from a determination of the composition of the reaction mixture as a function of time. The present paper develops a procedure for the evaluation of the integral equations of a dual pathway reaction in order to obtain the rate constants for the individual steps.

An automatic amino acid analyzer (Beckman–Spinco Model 120B) was used to obtain the concentrations of the octapeptide, heptapeptide, alanine, and desoctapeptide B chain (B-8)[1] as a function of time during the tryptic hydrolysis of B chain (Figure 1). All four kinetic constants were available from the data but the constant, k_{21}, could not be determined with accuracy. This was because the rate of cleavage of the arginyl bond (k_1) was so much greater than the lysyl bond (k_2) that only a minute amount of desalanine B chain (B-1)[1] appeared in the reaction mixture. An independent experiment was performed using B-1 as starting substrate to check the values for k_{21}.

Experimental Section

Materials. Bovine zinc insulin was the product of Eli Lilly and Co. (lot no. 836550). TPCK-trypsin[1] was prepared by treating twice-crystallized, salt-free, lyophilized trypsin from Worthington Biochemical Corp. (lot no. 6224) with L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone (Schoellmann and Shaw, 1963) by a modification (Wang and Carpenter, 1965) of the procedure of Kostka and Carpenter (1964). Trypsin treated with TPCK was used in order to minimize the possibility of nontryptic cleavage of peptide bonds by chymotrypsin which is a frequent contaminant of trypsin preparations. The *heptapeptide*, Gly-Phe-Phe-Tyr-Thr-Pro-Lys (all L isomers) was a sample synthesized by Shields and Carpenter (1961). The *octapeptide*, Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala, was isolated from the partial tryptic hydrolysate of oxidized beef insulin by a chromatographic procedure described previously (Wang and Carpenter, 1965). N^{α}-Carbobenzoxy-L-lysine-*p*-nitrophenyl ester hydrochloride was a product of Cyclo Chemical Corp. (lot no. M-2034). N^{α}-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE)[1] and N^{α}-benzoyl-L-arginamide hydrochloride (BAA)[1] were purchased from Mann Research Laboratories, Inc. (lots E1783 and E1892). N^{α}-Benzoyl-L-lysinamide hydrobromide (BLA)[1] was synthesized by a procedure similar to that given by Hofmann and Bergmann (1939) for the hydrochloride except that the decarbobenzoxylation was effected by HBr in acetic acid (Greenstein and Winitz, 1961) instead of catalytic hydrogenation. The product was crystallized from anhydrous methanol with dry ether, mp 199–201°, $[\alpha]_D^{27}$ −1.5°, and $[\alpha]_{300}^{27}$ −10.5° (*c* 1, methanol).

Anal. Calcd for $C_{13}H_{20}BrN_3O_2$: C, 47.24; H, 6.06; Br, 24.2; N, 12.7. Found: C, 47.23; H, 5.98; Br, 23.2; N, 12.3.

Pyridine was purified before use by a procedure given by Dixon (1956). The *oxidized B chain of insulin* was prepared by a method similar to that of Sanger (1949) and further purified by a chromatographic procedure described below. Amino acid analysis of the material by the procedure of Spackman *et al.* (1958) after hydrolysis in 6 N HCl at 120° for 6 hr in an evacuated sealed tube gave the following results normalized to one residue of aspartic acid: $Lys_{0.98}His_{2.04}Arg_{1.01}CySO_3H_{2.02}Asp_{1.00}Thr_{0.93}Ser_{0.94}Glu_{3.01}Pro_{1.04}Gly_{3.06}Ala_{2.03}Val_{2.90}Leu_{4.04}Tyr_{1.95}Phe_{2.93}$. Complete digestion of the material with trypsin and analysis for

the heptapeptide (Gly-Phe-Phe-Tyr-Thr-Pro-Lys) by the procedure of Wang and Carpenter (1965) gave a value of 1.02 moles of heptapeptide/mole of oxidized B chain (molecular weight 3663). *Desalanine B chain* (B-1) was prepared by treating oxidized insulin B chain (98 mg in 45 ml of pH 9.2 Tris–Cl buffer) with carboxypeptidase A (Worthington Biochemical Corp., 6125) for 2 hr at room temperature (enzyme:substrate, 1:25, w/w). The desalanine compound was precipitated with acetone and had the following amino acid composition based on aspartic acid: $Lys_{1.08}His_{1.98}Arg_{1.00}CySO_3H_{1.98}Asp_{1.00}Thr_{1.04}Ser_{1.00}Glu_{3.05}Pro_{1.02}Gly_{2.90}Ala_{1.04}Val_{2.75}Leu_{3.86}Tyr_{1.95}Phe_{2.80}$.

Determination of the normality of the trypsin solution was carried out by a titration method given by Bender *et al.* (1965a,b), using N^{α}-carbobenzoxy-L-lysine-*p*-nitrophenyl ester as titrant. The normalities for TPCK-trypsin and Worthington trypsin were found to be 68 and 64% of those calculated on weight basis, assuming the molecular weight of trypsin was 24,000 (Laskowski, 1955). In all calculations involving the concentration of the enzyme, the concentration refers to the active concentration as determined by this method.

Assay of the Trypsin Activity. A stock solution of TPCK-trypsin (20 mg in 5 ml of 0.001 N HCl containing 0.001 M $CaCl_2$) was prepared and used throughout all the experiments except those with desalanine B chain. Its catalytic activity was assayed by a method similar to that of Schwert and Takenaka (1955) using BAEE as a substrate and measuring the increase in absorbancy at 253 mμ with Cary 14 recording spectrophotometer. Bender and Kaiser (1962) defined the activity of trypsin as absorbancy change per second per milligram of trypsin per milliliter. The values obtained for TPCK-trypsin and Worthington trypsin were 0.678 and 0.626 absorbance unit/sec per mg of trypsin per ml, respectively (pH 8.0, 0.05 M potassium phosphate buffer, at 25°). This corresponds to 1.38 and 1.35×10^3 moles of substrate turned over/mole of enzyme per min based upon the value of $\Delta\epsilon = 1048\ M^{-1}\ cm^{-1}$ at 253 mμ (measured directly from a sample before and after complete hydrolysis of BAEE) and the absolute normality of the enzyme determined by the titration method.

pH Stability of Trypsin. The effect of pH on the stability of trypsin was studied under conditions similar to those employed for the hydrolysis of the oxidized B chain. Aliquots (50 μl) of the TPCK-trypsin stock solution (20 mg/5 ml) were pipetted into 45 ml of 0.01 M $CaCl_2$ solution maintained at 30° and at the desired pH values in the pH-Stat. Samples were removed at various time intervals and assayed for their trypsin activity on BAEE by the procedure described above. The stability of TPCK-trypsin at different pH values is summarized in Table I. The buffer used in the enzyme assay was 0.05 M ammonium bicarbonate instead of potassium phosphate in order to avoid the formation of turbidity due to the insolubility of calcium phosphate.

Conditions for the Reaction of Trypsin with Oxidized B Chain of Insulin. All of the reactions were performed in a jacketed vessel (50 ml) maintained at constant temperature by circulating water. The oxidized B chain was dissolved directly in 42 ml of 0.01 M $CaCl_2$ inside the digestion chamber. The solutions were stirred magnetically and maintained at the desired pH by a Radiometer TTTlc pH-Stat using 1 N NaOH in the syringe. After temperature and pH equilibration were attained (30–40 min), the reaction was started by adding the desired volume of enzyme from a micropipet. Samples (3 or 4 ml) were withdrawn at various time intervals and pipetted directly into vials containing enough 4 N HCl to bring the pH to about 2. The samples were stored frozen until subjected to chromatographic analysis.

TABLE I: Stability of TPCK-Trypsin at 30° at Different pH Values.[a]

Time (hr)	pH				
	5.0	8.0	9.0	10.0	10.7
	% of Original Activity				
1	...	...	...	92	85
2	99	...	98	90	81
4	103	...	105	87[b]	74
8	100	100	99	78	...
12	...	...	...	68	40
20	87	92	84[c]	...	...

[a] The enzyme concentration used was 1.36×10^{-7} M in 0.01 M $CaCl_2$ solution. [b] 3.3 hr. [c] 24 hr.

Analysis for the Products Formed by the Action of Trypsin on the Oxidized B Chain and Desalanine B Chain of Insulin. The samples were chromatographed on an amino acid analyzer (Beckman–Spinco Model 120B) using the 0.9 × 15 cm column packed with type 50A resin. For the products from B chain, elution was performed at a flow rate of 30 ml/hr using a linear gradient obtained by running 2.0 M pyridinium acetate buffer at pH 6.2 (120 ml) into a 0.1 M pyridinium acetate buffer at pH 3.5 (120 ml). The location of the various components of the digestion mixture is shown in Figure 2. The amount of each component was determined from the peak height–band width method (HW method of Spackman *et al.*, 1958) using the HW values measured on the pure compounds. Direct measurements were made for the amount of alanine (Ala), desoctapeptide B chain (B-8), heptapeptide (Hept), and octapeptide (Oct) in the reaction mixture. The amount of desalanine B chain (B-1) formed, as well as the amount of unreacted B chain (B) remaining in each sample, was calculated from the relationships

$$(B\text{-}1) = (Ala) - (Hept) \quad (1)$$

$$(B) = (B)_0 - (Ala) - (Oct) \quad (2)$$

where $(B)_0$ equals the original amount of oxidized

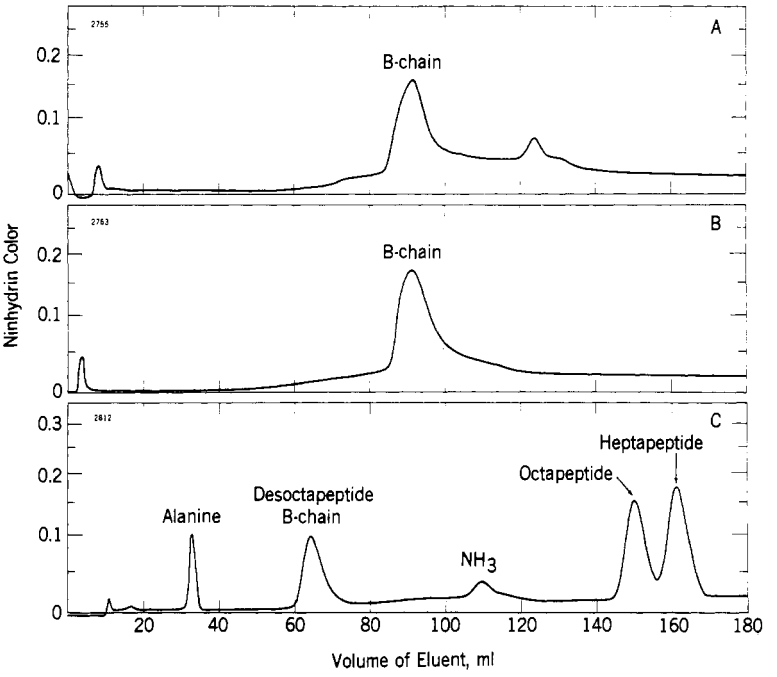

FIGURE 2: Ion-exchange chromatography by a linear gradient of pyridinium acetate buffer (pH 3.5, 0.1 M; pH 6.2, 2.0 M; 120 ml each) of (A) oxidized B chain (2.5/mg) prepared by Sanger's (1949) method, (B) the material in A after purification by the chromatographic procedure, and (C) the tryptides obtained from B chain (2 mg) by trypsin action.

B chain added as substrate. The amount of B-8 measured was in good agreement with the value calculated from the relationship

$$(B\text{-}8) = (Oct) + (Hept) \qquad (3)$$

The analysis of the products obtained from the action of trypsin on desalanine B chain was carried out by a procedure essentially identical with the heptapeptide assay method (Wang and Carpenter, 1965) except that the analyzer was equipped with an accelerated system (40-ml/hr flow rate, 1.45 M pyridinium acetate at pH 6.2) and an expanded range card for increased sensitivity.

Measurement of the Relative Rates of Hydrolysis of BAA and BLA by Trypsin. The rate of hydrolysis of these two compounds was followed spectrophotometrically at 253 mμ ($\Delta\epsilon$ = 750 M^{-1} cm^{-1}). The difference spectrum of the arginine derivative before and after hydrolysis was identical with that of the lysine derivative. Neither difference spectrum was affected by a change in pH from 8 to 11. Reactions were carried out in 0.05 M buffers (sodium phosphate, pH 8.0; sodium borate, pH 9.0; sodium carbonate, pH 10, 10.7, and 11.0) using a 1-cm cuvet with 5.0×10^{-4} M substrate concentration at 30°.

Optical Rotatory Dispersion. The measurements were made with Cary 60 spectropolarimeter on unbuffered solutions (1.4×10^{-4} M) at pH 8.0 from 600 to 200 mμ using 0.5-, 0.05-, and 0.005-dm cells.

Calculations

Derivations. When the initial concentration of the substrate is well below the K_m of the enzyme for that substrate, the rate of reaction is directly proportional to substrate and enzyme concentrations. Although we have no direct measurement of K_m of these substrates, we have assumed in the following derivations that under the conditions employed (initial substrate concentration at 2.04×10^{-4} M or less) we are well below the saturation point of the enzyme. Under such conditions, at a constant enzyme concentration, the rate of hydrolysis should be first order with respect to substrate concentration. Also, since the concentration of the substrate is quite low, competition between two susceptible bonds and intermediate products for the enzyme may be ignored. The actual results obtained are in good agreement with these assumptions.

In Figure 1, the possible routes of attack of trypsin on the oxidized B chain of insulin are outlined. The symbols k_1, k_2, k_{12}, and k_{21} refer to the pseudo-first-order rate constants (obtained at constant enzyme concentration) where the subscripts indicate the bonds being hydrolyzed; 1, attack at arginyl bond of intact B chain; 12, attack at the lysyl bond on octapeptide; 2, attack at lysyl bond of intact B chain; and 21, attack at the arginyl bond on the desalanine B chain. The derivations are made in terms of the pseudo-first-order rate constants which are related to the second-order rate constants (k_1' and k_2', etc.) by dividing by the enzyme concentration (E_0).

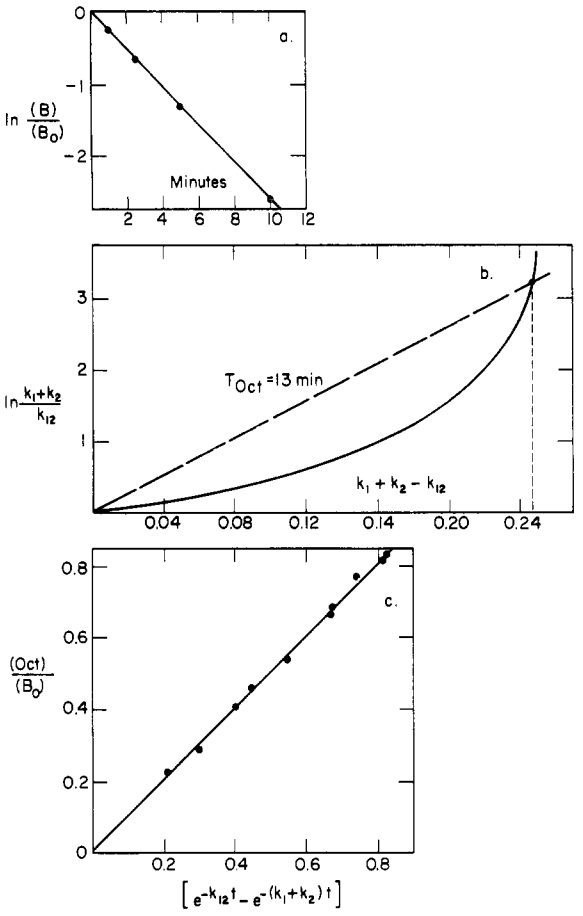

FIGURE 3: Graphic evaluation of the kinetic constants from the experimental data, (a) $(k_1 + k_2)$ from the slope, (b) k_{12} from the intercept, and (c) k_1 from the slope of the plot. (Solved for the experiments shown in Figure 4a.)

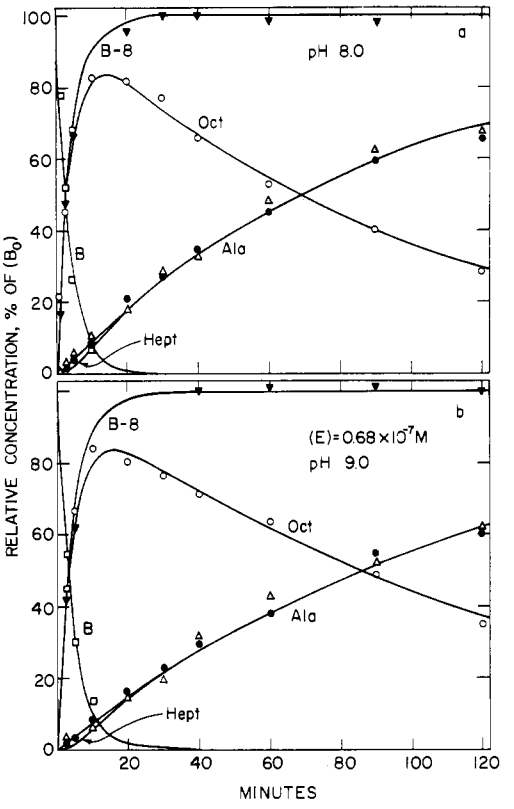

FIGURE 4: Results of the action of TPCK-trypsin (0.68 $\times$ 10^{-7} M) on oxidized bovine insulin B chain (1.43 $\times$ 10^{-4} M) at 30° with (a) pH 8.0 and (b) pH 9.0. The points are experimental and the lines are calculated values with k_1 = 0.254, k_2 = 0.0099, k_{12} = 0.0102, and k_{21} = 0.40 min^{-1} for a; and k_1 = 0.239, k_2 = 0.010, k_{12} = 0.0088, and k_{21} = 0.40 min^{-1} for b.

$$k_1/(E_0) = k_1'; \text{ etc.} \quad (4)$$

During the attack of trypsin on the oxidized B chain, the various reactions taking place can be formulated as following

$$-\frac{d(B)}{dt} = (k_1 + k_2)(B) \quad (5)$$

$$\frac{d(Oct)}{dt} = k_1(B) - k_{12}(Oct) \quad (6)$$

$$\frac{d(Hept)}{dt} = k_{12}(Oct) + k_{21}(B\text{-}1) \quad (7)$$

$$\frac{d(B\text{-}1)}{dt} = k_2(B) - k_{21}(B\text{-}1) \quad (8)$$

$$\frac{d(Ala)}{dt} = k_2(B) + k_{12}(Oct) \quad (9)$$

$$\frac{d(B\text{-}8)}{dt} = k_1(B) + k_{21}(B\text{-}1) \quad (10)$$

From eq 5;

$$(B) = (B_0)e^{-(k_1+k_2)t} \quad (11)$$

where (B_0) is the concentration of the B chain at zero time. When the value of (B) in eq 11 is substituted into eq 6, the resulting equation may be integrated (Esson, 1866; Long and Truscott, 1963; Briggs and Lipsky, 1965) to give (Oct) as a function of (B_0) and time t

$$(Oct) = (B_0)\left(\frac{k_1}{k_1 + k_2 - k_{12}}\right)[e^{-k_{12}t} - e^{-(k_1+k_2)t}] \quad (12)$$

By a similar procedure, one can obtain from eq 8 and 11

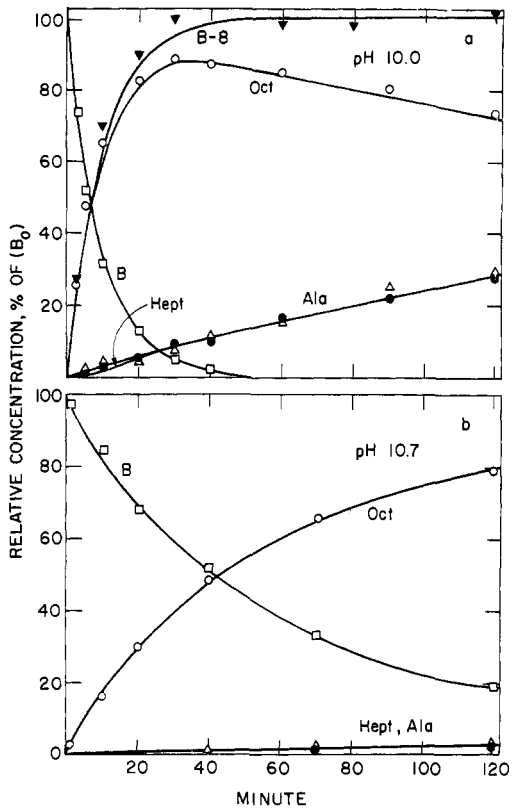

FIGURE 5: Results of the action of TPCK-trypsin on oxidized B chain of bovine insulin. Conditions were the same as Figure 4 except that at (a) pH 10.0 and (b) pH 10.7. The points are experimental and the lines are calculated values with $k_1 = 0.113$, $k_2 = 0.00334$, $k_{12} = 0.00269$, and $k_{21} = 0.25$ min^{-1} for a; and $k_1 = 0.0155$, $k_2 = 0.0003$, $k_{12} = 0.0003$, and $k_{21} = 0.015$ min^{-1} for (b).

$$(\text{B-1}) = (\text{B}_0)\left(\frac{k_2}{k_1 + k_2 - k_{21}}\right)[e^{-k_{21}t} - e^{-(k_1+k_2)t}] \qquad (13)$$

When eq 12 is differentiated with respect to t, and the derivative d(Oct)/dt is set equal to 0, one obtains a relationship between the time of the appearance for the maximum concentration of octapeptide (T_{Oct}) and the rate constants

$$T_{\text{Oct}} = \frac{\ln (k_1 + k_2) - \ln (k_{12})}{k_1 + k_2 - k_{12}} \qquad (14)^2$$

The above equations give solutions for (B), (Oct), and (B-1) as a function of time in terms of the individual rate constants and (B_0). A solution for (Hept) can then be obtained from the equation for the conservation of mass

$$(\text{Hept}) = (\text{B}_0) - [(\text{B}) + (\text{Oct}) + (\text{B-1})] \qquad (15)$$

Evaluation of the Rate Constants from the Experimental Data. The approximate values for the rate constants were determined first by graphic methods and then the more precise values were determined by a computer program. From eq 11, a plot of ln [(B)/(B_0)] against t gives a straight line whose slope is equal to $-(k_1 + k_2)$ (see Figure 3a). The sum $(k_1 + k_2)$ was then used to determine k_{12} from eq 14. To make this calculation, T_{Oct} was estimated from a plot of (Oct)/(B_0) *vs.* t (see Figures 4 and 5). The value of k_{12} was then determined from a plot of ln [$(k_1 + k_2)$ k_{12}] *vs.* $(k_1 + k_2 - k_{12})$ for various assumed values of k_{12}. The value of k_{12} was obtained from the intercept of this plot with a line of slope equal to T_{Oct} (Figure 3b). With k_{12} and the sum $(k_1 + k_2)$ at hand, the individual values of k_1 and k_2 were obtained by the use of eq 12. The experimentally determined ratios of (Oct)/(B_0) at different times were plotted against $[e^{-k_{12}t} - e^{-(k_1+k_2)t}]$. The slope of the resulting line is equal to $k_1/(k_1 + k_2 - k_{12})$ (Figure 3c). Since the sum $(k_1 + k_2 - k_{12})$ is known, k_1 can be calculated from this slope. Once k_1 is known, k_2 can be calculated since $(k_1 + k_2)$ has been determined.

A FORTRAN program was then written for an IBM 7040/7094 computer to calculate the ratios of (B), (Ala), (B-1), (B-8), (Oct), and (Hept) to (B_0) for various rate constants according to the equations derived. Using the values of k_1, k_2, and k_{12} obtained from the graphic method and setting k_{21} equal to k_1 as starting trial values,[3] the rate constants were changed one at a time until the best fits of the experimental data and the theoretical curves were obtained. The best fit was determined by the least-mean-square method. The trial values of the rate constants were varied until a minimum for the following was obtained.

[2] A similar treatment of eq 13 gives a relationship for the time of the appearance for the maximum of (B-1) and the rate constants as: $T_{\text{B-1}} = [\ln (k_1 + k_2) - \ln (k_{21})]/(k_1 + k_2 - k_{21})$. In the present instance, however, this equation is not useful owing to the difficulties in the direct measurement of time ($T_{\text{B-1}}$) for the maximum concentration of B-1. It should also be noted that, from eq 6 and 8, the ratios of the rate constants and the concentrations of reacting species have the relationships of: k_1/k_{12} = (Oct)/(B) at T_{Oct}, and k_2/k_{21} = (B-1)/(B) at $T_{\text{B-1}}$, since at these particular times, the differentials d(Oct)/dt and d(B-1)/dt are equal to zero. Although these relationships would be useful in some other problems, they were not applicable to the present study owing to the large difference between the rates of hydrolysis of arginyl *vs.* lysyl bond which causes difficulties in direct determinations of (B) at T_{Oct} and $T_{\text{B-1}}$.

[3] The value k_{21} was not determined by the graphic procedure owing to experimental difficulties rather than theoretical reasons (*cf.* footnote 2). The starting trial values for k_{21} were thus made equal to k_1. Justification for this procedure follows from the fact that k_1 and k_{21} were the rate constants for the cleavage of the same arginyl bond on very similar peptide substrates (B and B-1) which differ in only one alanine residue that was distantly located from the bond being cleaved. The k_{21} values obtained after computer programming were of the same order of magnitude as k_1 values. Furthermore, the value for k_{21} determined by direct measurement of the hydrolysis of B-1 was in close agreement with k_1.

TABLE II: Effect of Initial Substrate Concentration, Enzyme Concentration, pH, and Temperature on the Second-Order Rate Constants for the TPCK-Trypsin-Catalyzed Hydrolysis of Oxidized B Chain of Bovine Insulin.

	Second-Order Rate Constants			Ratios	
	k_1'	k_2'	k_{12}'	k_1'/k_2'	k_{12}'/k_2'
	(10^5 l. mole^{-1} min^{-1})				
A.[a] Initial substrate concentrations at pH 9.0 and 30°					
2.06×10^{-4} M	33.6	1.46	1.21	23.0	0.83
1.43×10^{-4} M	35.0	1.47	1.30	23.8	0.88
0.68×10^{-4} M	33.0	1.60	1.25	20.6	0.78
B.[b] Enzyme concentration, at pH 9.0 and 30°					
1.09×10^{-7} M	32.4	1.40	1.22	23.2	0.87
0.68×10^{-7} M	35.0	1.47	1.30	23.8	0.88
0.27×10^{-7} M	33.4	1.43	1.34	22.6	0.93
C.[a,b] pH at 30°					
8.0	37.4	1.48	1.50	25.2 (2.28)[c]	1.00
9.0	35.0	1.47	1.30	23.8 (2.61)	0.88
10.0	16.6	0.49	0.39	34.5 (3.31)	0.81
10.7	2.28	0.044	0.044	51.8 (7.12)	1.00
11.0	...	...	...	... (15.5)	
D.[a,b] Temperature (°C), at pH 9.0					
10	17.8	0.60	0.55	29.7	0.91
20	24.2	1.0	0.93	24.2	0.93
30	35.0	1.47	1.30	23.8	0.88
40	30.5	1.46	1.26	20.9	0.86

[a] Concentration of TPCK-trypsin equals 0.68×10^{-7} M. [b] Initial concentration of oxidized B chain equals 1.43×10^{-4} M. [c] Values in parenthesis are the ratios of initial velocities for the cleavage of the arginyl bond to the lysyl bond in BAA and BLA (5.0×10^{-4} M).

$$\text{mean square} = [\Sigma_i \Sigma_j W_{ij}(\Delta_{ij})^2]/j \qquad (16)$$

where Δ_{ij} is the difference between the experimental and calculated values of ith species at the jth experimental point. The W_{ij} are the weights assigned to compensate for the difference in the accuracy of the various measurements. It was assumed that the accuracy of the measurements made by the amino acid analyzer was directly proportional to the amount of the substances present in the sample within the range where the experiments were carried out (optical density at 570 mμ, from 0 to 0.3). The mean-square procedure was quite sensitive to changes in the values of k_1, k_{12}, and k_2. In any one experiment, the minimum was sensitive to variation of these values by as little as 0.1%. This was not true for k_{21}. In the critical range near the minimum, values for k_{21} could be varied over a twofold range without being reflected in the minimum of the mean-square solution. The reproducibility of values for k_1, k_{12}, and k_2 in various experiments indicated that the over-all experimental error was reflected by a standard deviation of about 3%.

Results

The stability of the enzyme at 30° as a function of pH is shown in Table I. These results indicate that during the 2-hr time interval for the kinetic studies, the enzyme was completely stable at pH 8 and 9. However, there was a 10 and 20% drop in activity during this time interval at pH 10.0 and 10.7, respectively. No attempt was made to correct the kinetic data for this gradual inactivation of the enzyme at these high pH values. The inactivation at pH 10.0 is too small to have had any appreciable effect on the kinetic constants. The inactivation at pH 10.7 is more serious and as a result, less reliance can be put on the absolute values of the constants. However, the loss of enzyme during the reaction should have no effect on the relative values of the various constants determined at this pH.

The effect of initial substrate concentration on the progress of the reaction is shown in Table IIA. The second-order rate constants were practically independent of initial substrate concentration in the range of

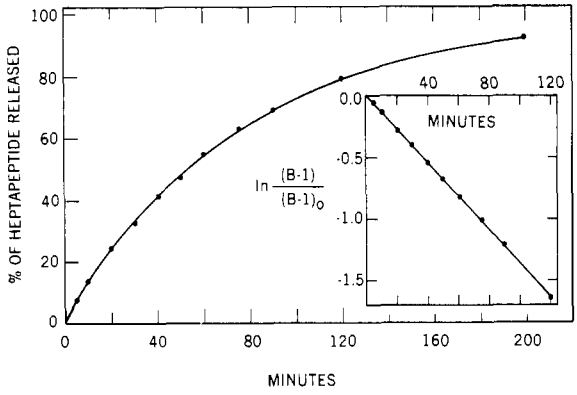

FIGURE 6: Results of the action of TPCK-trypsin (4.5×10^{-6} M) on desalanine B chain of bovine insulin (1.45×10^{-4} M) at 30° in borate buffer (0.05 M) at pH 9.0 which was 0.01 M in $CaCl_2$.

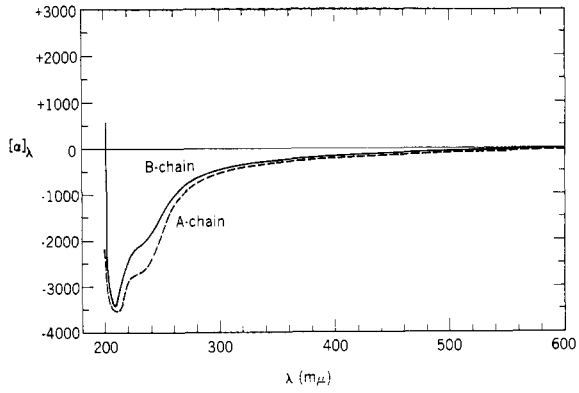

FIGURE 7: Optical rotatory dispersion of oxidized A and B chain of bovine insulin (1.4×10^{-4} M) at pH 8.0 unbuffered solution.

0.68–2.06 $\times 10^{-4}$ M. Therefore, the substrate concentration used in these studies was far enough below the K_m of the enzyme to avoid saturation effects and the reactions were first order with respect to substrate concentration.

The enzyme concentration was varied in order to obtain a further check on the conditions for the reaction. The second-order rate constants which take into consideration the concentration of the enzyme were practically unchanged during variation of the enzyme concentrations from 0.27 to 1.09 $\times 10^{-7}$ M (Table IIB).

The effect of pH on the reaction is shown in Figures 4 and 5 for pH values of 8, 9, 10, and 10.7. The second-order rate constants calculated from these data are shown in Table IIC. The maximum values for all rate constants were obtained at pH 8. These values decreased on increasing the pH above 8, but the magnitude of this effect was much more intense at pH 10 and above. The relative rate of splitting of the arginyl as compared to the lysyl bond on the intact B chain (k_1'/k_2') was about 25:1 at pH 8 and 9, but became increasingly higher at pH 10 (35:1) and 10.7 (52:1). The rate of splitting of the lysyl bond in octapeptide (k_{12}') was approximately equal to that of the same bond in the intact B chain (k_2') at all pH values.

The effect of temperature on the rate of hydrolysis of the oxidized B chain by trypsin was studied at 10, 20, 30, and 40°, at pH 9.0 with the results shown in Table IID. From a plot of the logarithms of the rate constants against the reciprocals of the absolute temperature,[4] the enthalpy of activation (ΔH^*) was calculated from the relationship $\Delta H^* = E_a - RT$. Values of 5.22 and 7.25 kcal/mole at 30° were found for ΔH^* of trypsin hydrolysis of arginyl bond and lysyl bond in the B chain. The free energy of activation (ΔF^*) and entropy of activation (ΔS^*) were calculated according to Dixon and Webb (1964) to give 11.1 and 13.0 kcal/mole and 19.5 and 19.1 eu, respectively, for these two bonds at 30°. There is 2 kcal/mole difference in both ΔF^* and ΔH^* for arginyl bond and lysyl bond. However, the entropies of activation for these two bonds are almost identical.

The hydrolysis of desalanine B chain (B-1) by trypsin was performed at pH 9.0, 30°, with the results shown in Figure 6. The second-order rate constant (k_{21}') was calculated to be 30.3×10^5 l. mole^{-1} min^{-1} which is very close to k_1' obtained under similar conditions (see Table II, $k_1' = 35.0 \times 10^5$ l. mole^{-1} min^{-1} at pH 9.0, 30°).

Influence of pH on relative rate of hydrolysis of BAA and BLA by trypsin was studied at various pH values, at 30°. The ratio of the initial velocities is given in Table IIC as a function of pH (in the parentheses). It can be seen that the ratio increases dramatically with pH especially above pH 10.

The optical rotatory dispersions of the A and B chains of oxidized insulin from 200 to 600 mμ at 27° and pH 8.0 are shown in Figure 7. The specific rotation ($[\alpha]_D^{27}$), and the a_0 and b_0 values of the Moffit equation (Moffit and Yang, 1956) were calculated to be −66°, −468, and −21 for A chain and −55°, −385, and −36 for B chain, respectively. The b_0 value along with the shape of the rotatory dispersion curves between 200 to 240 mμ (Blout *et al.*, 1962) indicate that the chains exist primarily as random coils under the conditions of measurement.

Discussion

In deriving the equations that were used to obtain the various velocity constants from the analysis of the products formed by the action of trypsin on the oxidized B chain of insulin, it was assumed that the reaction was first order with respect to substrate and enzyme

[4] The values obtained at 40° did not fall on the straight line defined by the values for 10–30° and were not included in making the calculations. This deviation from linearity could possibly be due to a change in conformation of the enzyme at temperatures above 30° (Anson and Mirsky, 1934; Massey *et al.*, 1966).

concentrations. The results obtained on variation of the enzyme and initial substrate concentrations indicated that the first-order assumption was valid under the conditions used in these experiments. Under all conditions, the arginyl bond of the B chain was attacked at a much faster rate than the lysyl bond. In most of the instances, better than 95% of the reaction followed the upper course of Figure 1, where the substrate was first hydrolyzed to octapeptide and then the octapeptide was degraded at a much slower rate to give heptapeptide and alanine. Less than 5% of the B chain was hydrolyzed *via* the lower pathway of Figure 1, involving primary attack at the lysyl bond to release alanine, followed by a secondary attack on the arginyl bond of desalanine B chain to give heptapeptide. Because of the large disproportionality of these two routes, it was not possible to obtain good values of k_{21} directly from the experiments using intact B chain as substrate. The independent experiment which made use of B-1 as substrate indicated that at pH 9 and 30°, the value for k_{21} determined directly was about the same as k_1. Since such a small fraction of the reaction follows the lower pathway of Figure 1, the theoretical curves for the progress of the reaction are relatively insensitive to the values for k_{21}. Such values may be varied over a twofold range without affecting the shape of the progress curves appreciably. In this sense the present system is not ideal for demonstrating the use of the two-pathway kinetic treatment developed in this paper.

In the previous studies performed on intact insulin, it was observed that alanine and heptapeptide appeared at about the same rate (Carpenter and Baum, 1962). This result is compatible with the present findings using B chain as substrate. The progress curves of the reactions (*cf.* Figures 4 and 5) show that except for the very early stages of the reaction, alanine and heptapeptide appear at about the same rate whenever the upper route of Figure 1 is the predominant pathway in the hydrolytic reaction. Thus, it would appear that with intact insulin as a substrate the hydrolysis of the arginyl bond also proceeds at a faster rate than that of lysyl bond.

At pH 8 and 9, the arginyl bond in B chain is cleaved at a rate about 25 times that of lysyl bond. The results of the experiments with model peptides in the present work as well as in the work of Hofmann and Bergmann (1941) at pH 7.5 indicated that when these residues were contained in similar peptides, there was only about a twofold advantage for the cleavage of the arginyl bond over lysyl bond in the pH range of 7.5–9.0. The greater disproportionality exhibited in the rates of cleavage of these two bonds in B chain as compared to model peptides may be attributed to several different factors. The B chain may possess a secondary or tertiary structure which results in a differential masking of the lysyl *vs.* the arginyl bond. The fact that the cleavage of the lysyl bond in octapeptide takes place at about the same rate as the lysyl bond in intact B chain together with the random coil structure indicated by optical rotatory dispersion measurements on the B chain are strong arguments against a highly organized secondary or tertiary structure. However, the possibility of hydrogen bonding between the phenolic group of Tyr_{26} and the ϵ-NH_2 of Lys_{29}, as suggested by Kurihara *et al.* (1965), which might render the lysyl bond less susceptible to trypsin is not eliminated. Nevertheless, it appears more likely that the disparity in rates can be largely attributed to the differing effects of the adjacent amino acids (Hill, 1965); the C-terminal Pro-Lys-Ala-OH sequence being more resistant to hydrolysis than the internally located Glu-Arg-Gly sequence.

Increasing the pH from 8 to 10.7 results in decreasing the rate of hydrolysis of both arginyl and lysyl bonds. This result is in agreement with the data upon model peptides in which a pH optimum of 7.6–8.0 has been reported (Laskowski, 1961). The effect of pH on rate was more pronounced for the lysyl bond than for the arginyl bond, especially at pH 10 or above. This difference in effect is reflected in the ratio of splitting of these two bonds (k_1'/k_2') which increases from 25:1 at pH 8 to 52:1 at pH 10.7. Preliminary experiments with model peptides, BAA and BLA, showed a similar divergency of the ratio at pH values greater than 10. At pH 10–11, the ϵ-NH_2 group of lysine is partially discharged whereas the guanidinium group of arginine still retains its full charge. So, the relative superiority of arginine to lysine residue for trypsin substrate at pH values higher than 10, as compared with 8, may well be attributed to the difference in the amount of positive charge carried by the side chains. However, the data do not rule out the possibility that the enzyme undergoes a conformational change at high pH resulting in a conformer with decreased ability to catalyze the hydrolysis of lysyl relative to arginyl bonds.

Although the rate of splitting of the arginyl bond in oxidized B chain at pH 10.7 is about 1/17 the rate at pH 8, the rate is still quite appreciable. Furthermore, as we have found, trypsin is remarkably stable at high pH values in the presence of Ca^{2+}. In our experiments, it retains 80% of its activity after 2 hr at pH 10.7 and 30°. The studies of Crewther (1953) indicate that trypsin is somewhat stable on exposure to even higher pH. These facts, combined with the differing effect of pH on the rates of cleavage of arginyl and lysyl bonds, suggest that tryptic digestion of proteins at high pH might be used to obtain preferential cleavage of arginyl bonds over lysyl bonds.

The effect of temperature on the rate constants at pH 9.0 was such that the ratio k_1'/k_2' increases upon decrease in temperature. This result reflects the fact that the over-all free energy of activation of lysyl bond cleavage was greater than that of arginyl bond.

In connection with our results on the effects of pH and temperature, it should be noted that Keil and Keilova (1964) did not detect any noticeable pH or temperature effect in their studies on the action of trypsin on oxidized B chain of insulin. Their studies involved only limited changes in pH (6.0 and 8.6) and temperature (20 and 37°). Furthermore, their

analytical techniques were not adequate for the determination of the rate constants. Consequently, it is doubtful that the effects of pH and temperature noted in our work could have been detected.

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References

Anson, M. L., and Mirsky, A. E. (1934), *J. Gen. Physiol. 17*, 393.

Bender, M. L., and Kaiser, E. T. (1962), *J. Am. Chem. Soc. 84*, 2556.

Bender, M. L., Kezdy, F. J., and Feder, J. (1965a), *J. Am. Chem. Soc. 87*, 4953.

Bender, M. L., Killheffer, J. V., and Roeske, R. W. (1965b), *Biochem. Biophys. Res. Commun. 19*, 161.

Bergmann, M. (1942), *Advan. Enzymol. 2*, 49.

Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.

Briggs, T., and Lipsky, S. R. (1965), *Biochim. Biophys. Acta 97*, 579.

Carpenter, F. H., and Baum, W. E. (1962), *J. Biol. Chem. 237*, 409.

Crewther, W. G. (1953), *Australian J. Biol. Sci. 6*, 597.

Dixon, H. B. F. (1956), *Biochem. J. 64*, 47P.

Dixon, M., and Webb, E. C. (1964), Enzymes, 2nd ed, New York, N. Y., Academic, p 150.

Esson, W. (1866), *Phil. Trans. Roy. Soc. London 156*, 216.

Greenstein, J. P., and Winitz, M. (1961), *in* Chemistry of the Amino Acids, Vol. 2, New York, N. Y., Wiley, p 1245.

Harris, J. I., and Li, C. H. (1952), *J. Am. Chem. Soc. 74*, 2945.

Hill, R. L. (1965), *Advan. Protein Chem. 20*, 37.

Hofmann, K., and Bergmann, M. (1939), *J. Biol. Chem. 130*, 81.

Hofmann, K., and Bergmann, M. (1941), *J. Biol. Chem. 138*, 243.

Keil, B., and Keilova, H. (1964), *Collection Czech. Chem. Commun. 29*, 2206.

Keilova, H., and Keil, B. (1964), *Abstr. 6th Intern. Congr. Biochem., IUB, 32*, 316.

Kostka, V., and Carpenter, F. H. (1964), *J. Biol. Chem. 239*, 1799.

Kurihara, K., Horinishi, H., and Shibata, K. (1965), *Protein, Nucleic Acid, Enzyme (Japan) 10*, 483, 759.

Laskowski, M. (1955), *Methods Enzymol. 2*, 26.

Laskowski, M. (1961), *in* Biochemists' Handbook, Princeton, N. J., Van Nostrand, p 301.

Laskowski, M., Jr., Leach, S. J., and Scheraga, H. A. (1960), *J. Am. Chem. Soc. 82*, 571.

Long, D. A., and Truscott, T. G. (1963), *Trans. Faraday Soc. 59*, 2316.

Massey, V., Curti, B., and Ganther, H. (1966), *J. Biol. Chem. 241*, 2347.

Moffit, W., and Yang, J. T. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 596.

Nicol, D. S. H. W. (1960), *Biochem. J. 75*, 395.

Plieninger, H., Arnold, L., Fischer, R., and Hoffmann, W. (1965), *Ber. 98*, 1774.

Sanger, F. (1949), *Biochem. J. 44*, 126.

Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2*, 252.

Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.

Shields, J. E., and Carpenter, F. H. (1961), *J. Am. Chem. Soc. 83*, 3066.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Wang, S. S., and Carpenter, F. H. (1965), *J. Biol. Chem. 240*, 1619.

Young, J. D., and Carpenter, F. H. (1961), *J. Biol. Chem. 236*, 743.