Kinetic Studies on the Action of Carboxypeptidase A on Bovine Insulin and Related Model Peptides*

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ABSTRACT: Several model peptides which have carboxyl-terminal amino acids related to those found in bovine insulin were synthesized and tested as substrates for the action of carboxypeptidase A over the pH range 6.0–9.0. The normalized initial velocities ($v/[E]_0$) for the action of the enzyme on the carbobenzoxyglycyl derivatives of L-alanine, L-glutamine, and L-asparagine were directly proportional to the substrate concentration over the substrate range 0.01–0.15 M in the pH range 6.5–9.0. The second-order rate constants (k) for these substrates exhibited maximum values at pH 7.0–7.5. Carbobenzoxyglycyl derivatives of L-glutamic acid and L-aspartic acid were much poorer substrates than the corresponding amides. The initial velocities for the action of carboxypeptidase A on these substrates had maximum values at pH 6.0 (or below) and fell off rapidly at pH values above 7.0. The difference in the pH optimum between the neutral and acidic amino acid residues explains in part the differences previously encountered in the pH profile for the carboxypeptidase-catalyzed hydrolysis of asparagine or aspartic acid from insulin and desamido-insulin, respectively. In studies on insulin the rate of release of carboxyl-terminal alanine (from the B chain) and of asparagine (from the A chain) was markedly increased as the concentration of the insulin was lowered from 10 mg/ml to 1 mg/ml in the pH range 7.5–9.0. The rate of release of both amino acids increased in going from pH 7.5 to 9.0 at all concentrations of insulin. Furthermore, increasing the salt concentration brought about a decrease in the rate of release of carboxyl-terminal amino acids from insulin. These results on insulin are in contrast to those predicted from studies of the action of carboxypeptidase on model peptides. However, there is a good correlation between conditions which bring about depolymerization of insulin (as judged by ultracentrifugal studies) and those which increase the rate of attack of carboxypeptidase on insulin. This correlation suggests that the action of carboxypeptidase on insulin is largely dependent on the degree of polymerization of the insulin.

Rotatory dispersion studies have been made of the products of digestion: desalanine-insulin and desalanine-desasparagine-insulin. They indicated that removal of both carboxyl-terminal residues results in an unfolding of the secondary structure of insulin. In contrast, removal of the alanine residue had little effect on the protein conformation. The results may be correlated with the biological activities of these two insulin derivatives. Desalanine-desasparagine-insulin was also found to be less aggregated than the parent insulin molecule while desalanine-insulin behaved like insulin in its sedimentation behavior.

Carboxypeptidase A has been used extensively as a tool for elucidating the primary structure of proteins, and much valuable information about its mode of attack on proteins has been accumulated (Davie *et al.*, 1959; Neurath, 1960). The present investigation stems from earlier work in this laboratory in which carboxypeptidase A was used to determine the difference in the primary structure between insulin and desamido-insulin (Slobin and Carpenter, 1963a,b). We were attracted to a study of the kinetics of the insulin–carboxypeptidase A system by several features which made it appear suitable for such an investigation. The hydrolytic reaction is relatively simple; only two peptide bonds are split by the enzyme: a carboxyl-terminal alanine from the B chain of insulin and a carboxyl-terminal asparagine (or aspartic acid) from the A chain (Slobin and Carpenter, 1963a,b). We were particularly interested in the effects of pH, substrate, and salt concentrations on the rate of release of these carboxyl-terminal amino acids of insulin and in comparing these results with those obtained on model peptides. For this purpose the carbobenzoxyglycyl derivatives of L-alanine, L-asparagine, L-aspartic acid, L-glutamine, and L-glutamic acid were prepared and subjected to the action of carboxypeptidase A at various pH values.

Experimental Section

Synthesis of Peptides. Analyses were performed by the Microchemical Laboratory, Department of Chemistry, University of California, Berkeley. All melting points were determined in open capillaries and are uncorrected.

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TABLE I: Properties of Carbobenzoxyglycyl Derivatives.

Carbobenzoxy-glycyl	Mp (°C)	$[\alpha]_D^{25}$ (degree)	Formula	C (%)		H (%)		N (%)	
				Theory	Found	Theory	Found	Theory	Found
L-Alanine	131–133[a]	−8.5[b]	$C_{13}H_{16}N_2O_5$	55.70	55.57	5.75	5.61	9.99	9.91
L-Asparagine	129–131[c]	+6.9[d]	$C_{14}H_{17}N_3O_6$	52.01	51.66	5.30	5.47	12.99	12.84
L-Aspartic acid[e]	176–178	+7.7[f]	$C_{26}H_{42}N_4O_7$	59.75	59.62	8.10	7.95	10.72	11.01
L-Glutamine	155–156	+1.97[d]	$C_{15}H_{19}N_3O_6$	53.42	53.33	5.68	5.88	12.45	12.69
L-Glutamic acid	157–158[g]	+3.9[h]	$C_{15}H_{18}N_2O_7$	53.26	53.27	5.36	5.49	8.28	8.29

[a] Clayton *et al.* (1957) reported mp 133°. [b] 2.5% in ethanol. [c] Leach and Lindley (1954) reported mp 129.5–131°. [d] 2.5% in dimethylformamide. [e] Cyclohexylammonium salt. [f] 2.5% in water. [g] Hofmann and Bergmann (1940) reported mp 160–162°. [h] 2.5% in ethanol at 25°.

With the exception of carbobenzoxyglycyl-L-alanine, all of the substrates were prepared by the carbobenzoxylation of the corresponding glycyl peptides. The dipeptides in turn were synthesized by chloroacetylation of the carboxyl-terminal amino acid, followed by ammonolysis of the chlorine group to give the glycyl peptide (Fischer and Otto, 1903; Greenstein and Winitz, 1961). Carbobenzoxyglycyl-L-alanine was synthesized by condensation of carbobenzoxyglycine with alanine ethyl ester by the mixed anhydride procedure (Vaughan and Osato, 1952) using isobutyl chlorocarbonate. The resulting ester was saponified to give the peptide acid. All derivatives were characterized by melting point, specific rotation, and analytical composition (Table I). Several of these have not been reported previously in the literature.

Carboxypeptidase A. Bovine carboxypeptidase A was obtained as a suspension of crystals (twice crystallized) in water from the Worthington Biochemical Corp. (lot No. 690). Since this enzyme was made by the method of Anson (1937a,b), it would be the form designated as carboxypeptidase A_γ by Neurath and coworkers (Sampath Kumar *et al.*, 1963). To destroy possible tryptic or chymotryptic activity, the enzyme (1 g) in 10% LiCl (50 ml) was treated with diisopropylfluorophosphate (0.6 ml of a 0.1 M solution in dry 2-propanol). The suspension was stirred overnight at 4° and the carboxypeptidase was crystallized according to the method of Allan *et al.* (1964). This preparation of carboxypeptidase was used throughout these studies. Aliquots of the crystalline suspension of the enzyme were dissolved in 10% LiCl, and the concentration of the enzyme was obtained from optical density measurements at 278 mμ using the value of 1.94 for 1-cm thick solution of 1 mg/ml (Vallee *et al.*, 1960). During the kinetic experiments the normalized initial velocity of the enzyme was determined daily on carbobenzoxyglycyl-L-phenylalanine (0.02 M in the pH 7.5 buffer at 30°). An average of 25 such determinations gave a value of $4.10 \pm 0.5 \times 10^3$ mole of substrate reacting per mole of enzyme in 1 l. of solution per minute.

Insulin. Bovine zinc insulin was supplied by Eli Lilly and Co. (lot PJ 4812). It was converted to the zinc-free hydrochloride by precipitation from 0.25 N HCl solution with acetone (Carpenter, 1958). The concentration of insulin in solution was obtained from optical density measurements at 277 or 280 mμ. Triplicate determinations of the extinction coefficients for samples of the insulin hydrochloride which had been dried at 100° *in vacuo* over phosphorus pentoxide for 3 hr and dissolved in buffer at pH 8.0 gave mean values of 0.909 ± 0.024 and 0.990 ± 0.028 for 1-cm thick solutions of 1 mg/ml at 280 and 277 mμ, respectively.

Buffers and Assay Solutions. All pH measurements were made with a Radiometer pH meter, type PHM4c (Radiometer, Copenhagen, Denmark). In experiments involving the model peptides as substrates a mixed buffer consisting of 0.025 M Tris (Sigma 121), 0.025 M ethylenediamine (Matheson Coleman and Bell), and 0.5 M LiCl was used. In the kinetic studies on insulin the LiCl was omitted unless otherwise noted. Since the pK values of amine buffers change markedly with temperature (Bates, 1958), the solutions were adjusted so as to give the desired pH at 30°.

Spectrophotometric Assay on Model Peptides. The cell holder as well as the cell compartment of the Cary Model 14 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif.) was thermostated at 30 ± 0.2°. In most cases, the path length of the cuvet was reduced from 1 to 0.1 or 0.05 cm by the introduction of quartz inserts. The rate was measured as a decrease in absorption at 225 mμ for a period of 10–15 min with the enzyme concentration adjusted so that less than 10% reaction occurred in this interval. In order to obtain a positive slope on the trace, the signals from the light beam of the spectrophotometer were reversed so that the normal reference signal originated from the reaction mixture. In certain cases, when it was necessary to use large amounts of enzyme in the reaction cell, a solution of lysozyme was added to the reference cell in order to cancel the absorption due to the enzyme in the reaction cell. For this purpose crystalline lysozyme was dissolved in distilled water to give a solution containing 10 mg/ml. The appropriate amount of protein was determined from the fact that the extinction of carboxypeptidase is 0.7 times the value for lysozyme on a

weight basis at 225 mμ in pH 7.5 buffer. The lysozyme (twice crystallized) was obtained from Worthington Biochemical Corp. (lot No. 616).

The initial velocity of the reaction (ΔE/min) was determined from the slope of the linear trace of extinction *vs.* time. All substrates exhibited a linear rate of hydrolysis with respect to time over the pH range studied. Duplicate determinations were made at every substrate concentration, and the mean value was used to calculate the initial velocity. The value of ΔE/min was corrected to a 1-cm thick solution and was multiplied by the *reciprocal* of the difference between the molar extinction coefficients of the substrate and their enzymatic hydrolysis products to give the moles of substrate reacting per liter of solution per minute. The differences in the molar extinctions between substrates and their enzymatic hydrolysis products were 169, 216, 206, and 764 M^{-1} cm^{-1} for the carbobenzoxyglycyl derivatives of alanine, asparagine, glutamine, and phenylalanine, respectively. These values did not change between pH 6 and 9.

Product Assay on Model Peptides. Stock solutions of the substrate (1.0 M) were prepared at the desired pH in 0.5 M LiCl. Aliquots (100 μl) were added to either 0.8 or 8.9 ml of buffer at the desired pH. After the solutions had been equilibrated at 30°, carboxypeptidase (3.4 mg/ml in 10% LiCl) was added (0.1 ml to 0.9 ml of substrate and 1.0 ml to 9.0 ml of substrate solution) to give reaction mixtures which were 0.1 and 0.01 M in substrate. After incubation for 30 min at 30°, the reaction was stopped by the addition of a known volume of HCl so as to lower the pH to between 1 and 2. Acidified digests were stored at −10° until aliquots were removed for amino acid analyses on the automatic amino acid analyzer (Spackman *et al.*, 1958). Control experiments were performed (substrate plus buffer and enzyme plus buffer), and corrections for self-digestion of carboxypeptidase or trace impurities in substrates were made where necessary. Velocities were calculated directly from the amounts of amino acid liberated (moles of amino acid per minute per mole of enzyme per liter).

Treatment of Data for Model Peptides. For each substrate at each pH value the initial velocity (v) was measured as a function of substrate concentration. The initial velocity was normalized to the enzyme by dividing by the molar concentration of the enzyme to give the normalized initial velocity ($v/[E]_0$) in units of min^{-1}. A value of 34,300 g/mole was used for the molecular weight of the carboxypeptidase A (Smith and Stockell, 1954). For each pH, the values of $v/[E]_0$ were plotted against substrate concentration ([S]). When such a plot gave a straight line, the second-order rate constant (k of eq 1) was calculated from the slope of the line. Equation 1 defines the situation when substrate concentrations are small relative to K_m.

$$v/[E]_0 = k_{+2}[S]/K_m = k[S] \qquad (1)$$

When the substrate concentration was of the same magnitude as K_m, $v/[E]_0$ was plotted against $v/[E]_0[S]$ according to Eadie (1942) to give $-K_m$ from the slope and k_{+2} from the intercept. The symbols have the meanings recommended by the Commission on Enzymes (Florkin and Stotz, 1964).

Kinetic Studies on Insulin. The rate of release of alanine and asparagine by the action of carboxypeptidase A on insulin was measured at pH values of 7.5, 8.0, and 9.0 at a temperature of 30° and at two concentrations of insulin: 10 mg/ml (designated *high*) and 1 mg/ml (designated *low*). To study the kinetics of alanine release at high concentration, 5 ml of a solution containing 10 mg/ml of insulin in the mixed buffer at the appropriate pH was temperature equilibrated in a water bath at 30°. Carboxypeptidase (10 μl of a stock solution containing 7.91 mg/ml in 10% LiCl) was added with rapid mixing. Aliquots (1 ml) of the digestion mixture were removed at various time intervals (generally 5, 10, 20, and 40 min) and pipetted directly into a volumetric flask (5 ml) containing a few milliliters of 0.1 N HCl. This lowered the pH to between 2 and 3 and stopped enzyme action. The digests were made up to a volume (5 ml) with 0.1 N HCl and stored at −10° until amino acid analyses were performed by the method of Spackman *et al.* (1958).

To study the kinetics of alanine release at low concentration, a series of tubes each containing 10 ml of an insulin solution (1 mg/ml in the mixed buffer at the appropriate pH) were incubated with enzyme (50 μl of a stock solution containing 0.442 mg/ml). At various time intervals 1 N HCl (1 ml) was pipetted directly into one of the digestion mixtures. Each time interval thus represents a separate digestion.

Asparagine release was measured in an identical fashion except that the amount of enzyme added varied. At high insulin concentration 250 μl of enzyme stock solution containing 7.91 mg/ml was added; at low insulin concentration 20 μl of the same stock solution was used. The initial enzyme concentrations were chosen so that less than 40% hydrolysis of the carboxyl-terminal residue of interest was released in the time interval studied.

Appropriate blank determinations were performed. The enzyme preparation was found to be free of contaminating amino acids. There was no significant self-digestion of enzyme during the brief hydrolysis period.

The rate of hydrolysis at both high and low insulin concentrations was treated as a first-order reaction. The data were fitted to the following equation

$$\frac{\log (a)/(a - x)}{[E]_0} = kt/2.3 \qquad (2)$$

where a is the starting concentration of insulin, x is the amount of product (alanine or asparagine) formed in time t, k is the rate constant, and $[E]_0$ is the initial enzyme concentration in moles/liter using a value of 34,300 g/mole for the molecular weight of carboxypeptidase A (Smith and Stockell, 1954). The expression $a - x$ was obtained by subtracting the moles of alanine (or asparagine) released in time t from the initial moles of insulin present. The monomer molecular weight (5733) was used to calculate a at low starting concen-

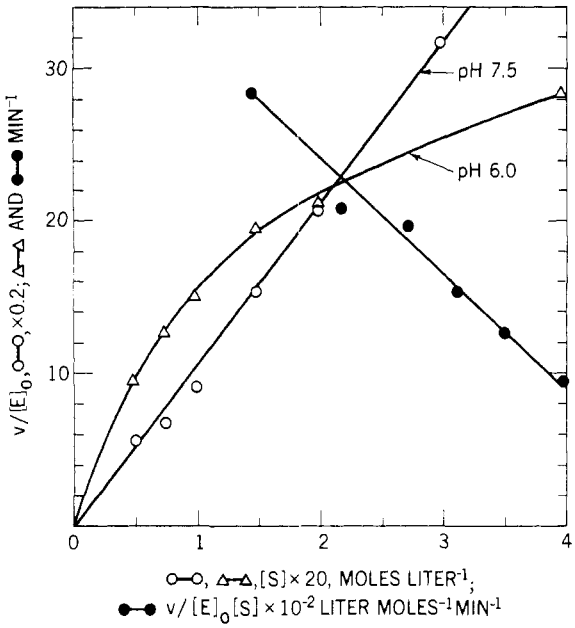

FIGURE 1: The normalized initial velocity ($v/[E]_0$) for the action of carboxypeptidase A on carbobenzoxyglycyl-L-glutamine at 30° in 0.025 M Tris–0.025 M ethylenediamine–0.5 M LiCl as a function of substrate concentration at pH 6.00 (Δ) and pH 7.50 (○) and as a function of $v/[E]_0[S]$ (Eadie plot) at pH 6.0 (●).

trations of insulin; the dimer molecular weight (11,466) was used for calculations at high initial insulin concentrations. In a number of experiments involving complete reaction of carboxypeptidase on insulin, this preparation of insulin (PJ4812) gave an average value of 90% carboxyl-terminal asparagine and 10% carboxyl-terminal aspartic acid. We had previously reported a slightly lower value (82%) for the carboxyl-terminal asparagine content of this preparation of insulin (Slobin and Carpenter, 1963b). In the calculations involving the rate of release of asparagine, the initial concentration of insulin was multiplied by 0.9. The rate constant, k, was obtained from a plot of log $[a/(a - x)]/[E]_0$ against t.

Sedimentation Coefficients. A Spinco Model E analytical ultracentrifuge fitted with a schlieren optical system was employed. All runs were made at 59,790 rpm using single sector Kel-F cells. In some cases 1° wedged windows were used to give two schlieren patterns simultaneously. Solutions were prepared in the mixed buffer described under Materials. The temperature was controlled during each run but varied from 20 to 25° from experiment to experiment.

Optical Rotatory Dispersion. All rotations and dispersions were measured in a modification of the Rudolph 200-S-80 spectropolarimeter. A description of the operation and use of the machine is given by Pour-El (1960). The light source was a General Electric tungsten lamp No. 2330. Dispersion could be obtained in the range 350 to 589 mμ.

A recent review of the theory of rotatory dispersion of proteins and polypeptides is given by Urnes and Doty (1961). Our data were fitted to a one-term Drude equation

$$[\alpha]_\lambda = \frac{A}{\lambda^2 - \lambda_0^2} \qquad (3)$$

where A is a complex constant depending in part upon the refractive index of the medium, $[\alpha]_\lambda$ is the specific rotation at wavelength λ, and λ_0 is a statistical average of the wavelengths of the optically active electronic transitions. The value of λ_c, which has been used as a measure of α-helical structure in proteins and synthetic polypeptides, was obtained from the slope of a plot of $[\alpha]_\lambda\lambda^2$ against $[\alpha]_\lambda$ (Yang and Doty, 1957).

Results

Spectrophotometric Assay. The difference in absorption between peptides and their hydrolysis products has been utilized to determine peptidase (Schmitt and Siebert, 1961) as well as carboxypeptidase activity (Young, 1959; Folk and Gladner, 1960). In the present instance the rate of splitting of the peptide bond in model peptides was followed by measuring the decrease in absorption at 225 mμ as the bond was hydrolyzed. The difference spectra were found to conform to Beer's law over the concentration range used here.

In order to delineate the experimental conditions, the initial velocity at the *lowest* substrate concentration to be employed was measured as a function of the enzyme concentration. This measurement was made for all substrates at all pH values. Only enzyme concentrations which fell on the linear portion of such a curve were employed in the assays.

At fixed enzyme and carbobenzoxyglycyl-L-phenylalanine concentration with the ionic strength maintained at 0.5 by appropriate addition of LiCl, the initial velocity was measured as a function of buffer ion concentration at pH 6.0 and 7.5. The rate was not affected by varying the concentration of Tris or ethylenediamine between 0.012 and 0.1 M.

Carbobenzoxyglycyl Derivatives of L-Alanine, L-Glutamine, and L-Asparagine. Carboxypeptidase A catalyzed hydrolysis of the carbobenzoxyglycyl derivatives of L-alanine, L-glutamine, and L-asparagine was determined by the spectrophotometric assay using 0.01–0.2 M substrate from pH 6 to 9. At pH values of 6.5 and above, the initial velocity was directly proportional to the substrate concentration over the range 0.01–0.15 M (Figure 1, ○). Substrate concentrations larger than 0.2 M were not investigated because of possible spurious effects from a change in the ionic strength of the medium. The second-order rate constants (k) calculated from the plot of normalized initial velocity ($v/[E]_0$) against substrate concentration are given in Table II. The carbobenzoxyglycyl derivatives of alanine and glutamine had about the same k values. Carbobenzoxyglycyl-L-asparagine was a much poorer

TABLE II: Kinetic Constants for the Carboxypeptidase A Catalyzed Hydrolysis of Various Carbobenzoxyglycyl-L-Amino Acids as a Function of pH at 30°.

	Carbobenzoxyglycyl Derivatives of						
	Alanine	Glutamine	Asparagine	Glutamic Acid		Aspartic Acid	
pH	k (liter moles^{-1} min^{-1})			$v/[E]_0$ (min^{-1} × 10^4)			
				0.01 M	0.1 M	0.01 M	0.1 M
6.00	900[a]	510[a]		4.7	30	6.2	38
6.50	750	580	12.4	2.7	24	4.5	38
7.00	1520	1010	20.9	0.8	20	3.1	28
7.50	1620	1070	(19.6)[b]	0.2	7.2	0.8	12
8.00	1040	920	16.2		1.2	0.5	3.4
8.50	590	510	11.9		0.5		1.7
9.00	340	410	7.6				

[a] Calculated from the relationship: $k = k_{+2}/K_m$; K_m = 0.08 M. [b] Values found at pH 7.30 and 7.70.

substrate; its rate constant was about $^1/_{50}$ that of the corresponding glutamine derivative.

At pH 6.0 the initial velocities for the carbobenzoxyglycyl derivatives of alanine and glutamine gave normal Eadie plots (Figure 1, ●) from which K_m values of about 0.08 M were obtained for both substrates. The asparagine derivative was such a poor substrate at pH 6.0 that it was not possible to obtain reliable kinetic data on this compound at this pH by the spectrophotometric assay.

The effect of pH on the k values was quite similar for the derivatives of alanine, glutamine, and asparagine. The rate constants increased from pH 6 to 7, exhibited a maximum in the pH range 7–7.5, and thereafter decreased.

Carbobenzoxyglycyl Derivatives of L-*Aspartic Acid and* L-*Glutamic Acid.* The carbobenzoxyglycyl derivatives of aspartic acid and glutamic acid were both such poor substrates for carboxypeptidase A that it was not possible to follow the reaction by the spectrophotometric method. A product assay was employed in which the liberated aspartic acid or glutamic acid was measured by the use of the amino acid analyzer. The use of this instrument rather than a procedure measuring the increase in total ninhydrin color of the reaction mixture upon cleavage of the peptide bond was necessitated by the background ninhydrin color value due to the large amount of enzyme required and to the use of amine buffers. The latter were used in an attempt to circumvent buffer ion inhibition. The high sensitivity of the instrument made it possible to obtain quite precise data from less than 1% reaction. However, the length of time needed for each analysis precluded an extensive determination of the effect of substrate concentration on the initial velocity. The initial velocity was measured at two substrate concentrations (0.01 and 0.1 M) over the pH range 6.0 to 8.5. The results (Table II) show that the initial velocity is not directly proportional to the substrate concentration over the range 0.01 to 0.1 M, thereby precluding a calculation of k values. The normalized initial velocities ($v/[E]_0$) of the various substrates can be compared at one substrate concentration (0.1 M) and pH value (6.5) to give 75, 58, 1.2, 0.0024, and 0.0038 min^{-1} for the carbobenzoxyglycyl derivatives of alanine, glutamine, asparagine, glutamic acid, and aspartic acid, respectively. Under these conditions there is a 300-fold difference in the $v/[E]_0$ values between the asparagine and aspartic acid derivatives and a 20,000-fold difference between the glutamine and glutamic acid derivatives.

The effect of pH on the normalized initial velocities was similar for the derivatives of glutamic acid and aspartic acid. Both exhibited a maximum value of $v/[E]_0$ at pH 6.0 to 6.5. In contrast to the corresponding amides the value decreased on going to pH values of 7.0 and above.

Insulin. The values shown in Table III are second-order rate constants which take into consideration the concentration of the enzyme. These constants were calculated on the basis that at low insulin concentration (1 mg/ml) and constant enzyme concentration the progress of the reaction followed the first-order rate law using a molecular weight of 5733 for insulin, and that at high insulin concentration (10 mg/ml) the first-order rate law was followed using the dimer molecular weight (11,466) for insulin. The justification for such a procedure is to be found in Figures 2 and 3 which show that at pH 8.0 the release of both alanine and asparagine follows first-order kinetics at low insulin concentration when the monomer molecular weight is used to make the calculations. At high insulin concentrations the dimer molecular weight allows a linear fit of the data. Similar observations were made at all pH values. It should be noted that in most cases the progress of the reaction was followed only during the release of the first 40% or less of the particular amino acid. The kinetics may very well not obey the first-order equation throughout the whole progress of the reaction. Moreover, the fact that the first-order rate law is followed

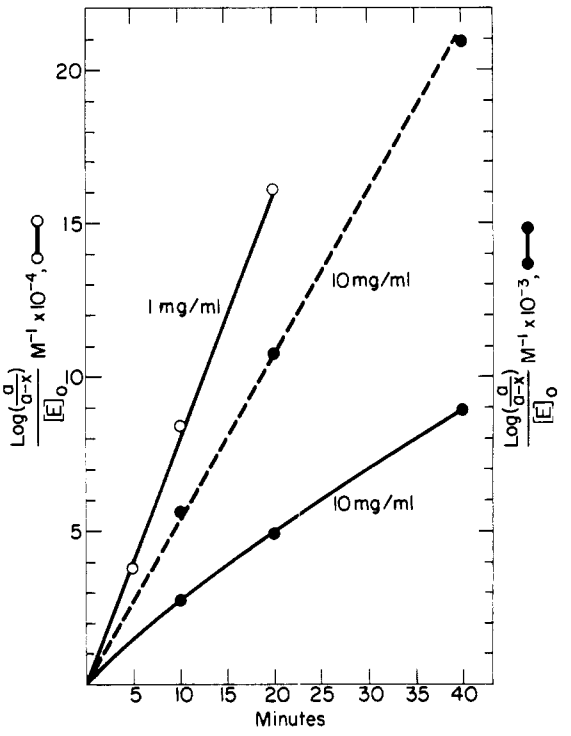

FIGURE 2: First-order plot for the release of carboxyl-terminal asparagine by the action of carboxypeptidase A on zinc-free insulin at pH 8, 30°, and at initial concentrations of insulin of 1 mg/ml (○) and 10 mg/ml (●) and with the monomer (solid line) or dimer (dashed line) molecular weight being used in the calculations.

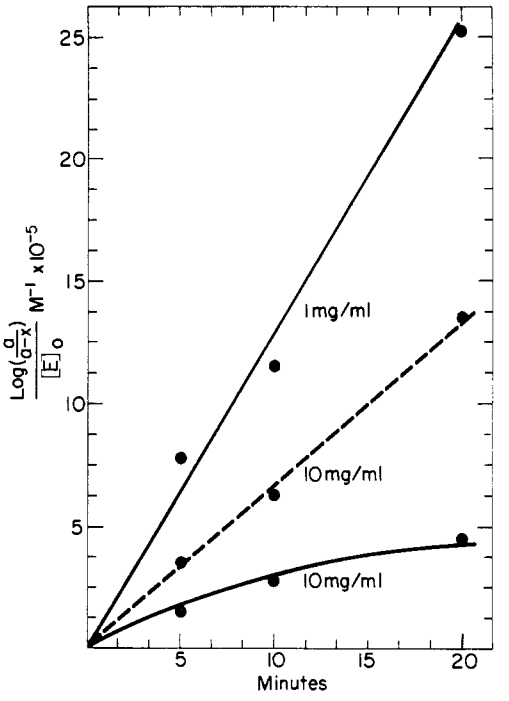

FIGURE 3: First-order plot for the release of carboxyl-terminal alanine by the action of carboxypeptidase A on zinc-free insulin at 30°, pH 8.0, and at initial concentrations of insulin of 1 mg/ml and 10 mg/ml with the monomer (solid line) or dimer (dashed line) molecular weight being used in the calculations.

at high concentrations when the dimer molecular weight is used in the calculations may be a fortuitous situation arising from a complex equilibrium between monomeric and polymeric forms of insulin. However, expressing the kinetic results as second-order rate constants has some pragmatic values. It allows for a numerical comparison of the effect of various factors such as salt concentration, insulin concentration, etc., on the kinetics of the carboxypeptidase A hydrolysis of insulin.

Increasing the substrate concentration at constant pH and enzyme concentration caused a decrease in the rate of release of carboxyl-terminal groups from insulin. This effect, which is reflected in the second-order rate constants (Table III), was observed at all pH values. Although the magnitude of the effect did not vary too greatly with pH, it was not the same for the release of alanine and asparagine. The rate of release of alanine decreased at most by one-half in going from insulin concentrations of 1 mg/ml to 10 mg/ml, while the rate of release of asparagine decreased to nearly $^1/_{20}$ over the same range.

Increasing the pH from 7.5 to 9.0 resulted in an increased rate of hydrolysis of both carboxyl-terminal groups (Table III). As judged by the second-order rate constants, the rate was about doubled in going from pH 7.5 to 9.0. The magnitude of this pH effect was about the same at high and low concentrations of insulin and for both of the carboxyl-terminal groups.

Increasing the LiCl concentration of the digestion mixture from about 0.02 to 0.30 M caused a pronounced

TABLE III: Effect of pH, Insulin, and Salt Concentrations on the Second-Order Rate Constants for the Carboxypeptidase A Catalyzed Hydrolysis of Alanine and Asparagine from Insulin at 30°.

	Alanine Release		Asparagine Release	
pH	10 mg/ml[a]	1 mg/ml[a]	10 mg/ml[a]	1 mg/ml[a]
	$k \times 10^{-3}$, liter moles^{-1} min^{-1}			
9.0	274	322	1.88[b]	26.7
8.0	152	304	1.21[b]	19.2
7.5	139	230	0.92[b]	12.7
7.5	129[c]			
7.5	90[d]			

[a] Insulin concentration. [b] Concentration of LiCl was 0.11 M. In all cases introduction of the enzyme also introduced some LiCl to the solution which unless otherwise noted was less than 0.02 M. [c] 0.15 M LiCl. [d] 0.30 M LiCl.

decrease in the second-order rate constant for the release of carboxyl-terminal alanine (Table III).

Sedimentation Results. The results of the effect of pH, insulin, and salt concentration on the rate of release of carboxyl-terminal amino acids from insulin by carboxypeptidase appeared to be related to the degree of polymerization of the molecule. In order to make a straightforward correlation with the degree of polymerization, we submitted insulin and insulin derivatives to sedimentation velocity studies under various conditions with the results shown in Table IV. The results are in agreement with those of previous workers, primarily Fredericq (1956), who investigated the effect of zinc, salt, and protein concentration on the sedimentation of insulin in the alkaline pH regions. We found that the Svedberg constants decreased with decreasing protein concentration and also upon increasing the pH from 7.5 to 9.0. Although our technical procedures did not allow us to get Svedberg values at the low insulin concentrations (1 mg/ml) used in the enzyme experiments, there was considerable drop in the s value in going from concentrations of 10 to 3.5 mg/ml. This was true at all pH values.

It was of interest to compare the s value of desalanine-desasparagine-insulin (the product of complete action of carboxypeptidase A on insulin) with insulin and desalanine-insulin. The results (Table IV) show that the desalanine-desasparagine-insulin is less associated than either insulin or desalanine-insulin. These latter two appear to have about the same behavior on sedimentation. In this connection it should be recalled that desalanine-desasparagine-insulin possesses very little biological activity while desalanine-insulin is quite potent (Slobin and Carpenter, 1963a,b).

Optical Rotatory Dispersion. The data obtained in the optical rotatory dispersion studies are shown in Table V. It can be seen that insulin possesses about the same λ_c value in the pH range 7.4 to 9.4, which is in agreement with the studies of Schellman (1958). Thus the dissociation of the molecule by pH that is indicated by the sedimentation studies is not reflected by optical rotatory dispersion measurements. Desalanine-insulin exhibits optical rotatory properties similar to those of insulin. Therefore, as judged by the technique of rotatory dispersion, there is no marked difference between the conformation of insulin and desalanine-insulin. Desalanine-desasparagine-insulin (λ_c 242 mμ) exhibited quite different dispersion properties than insulin (λ_c 260 mμ) and desalanine-insulin (λ_c of 258 mμ). The drop in λ_c value in going from insulin to desalanine-desasparagine-insulin may be attributed to some change in the conformation of the molecule (Urnes and Doty, 1961). At pH 8.0 and in the presence of 6 M guanidine, the λ_c of insulin has been shown to decrease to 220 mμ (Schellman, 1958). If this value is taken as a measure of the completely unfolded protein, then it would appear that desalanine-desasparagine-insulin has only a partially disrupted secondary structure. As noted previously (Table IV) desalanine-desasparagine-insulin sediments less rapidly than insulin or desalanine-insulin. This indicates that the disordered conformation of desalanine-desasparagine-insulin influences the ability of the molecule to aggregate.

TABLE IV: Svedberg Constants ($s_{20,w}$) of Insulin and Derivatives at Various Concentrations and pH Values.

Compound	pH	Concn (mg/ml)	$s_{20,w}$
Zinc-free insulin	7.5	10.0	2.97
Zinc-free insulin	7.5	3.3	2.41
Zinc-free insulin	9.0	10.1	1.94
Zinc-free insulin	9.0	3.4	1.65
Zinc-free insulin	9.0	10.1	2.04
Desalanine-desasparagine-insulin[a]	9.0	10.1	1.29
Desalanine-insulin[b]	9.0	5.2	1.47
Desalanine-desasparagine insulin	9.0	5.1	1.17
Desamido-insulin[c]	9.0	4.9	1.34

[a] Desalanine-desasparagine-insulin was purified by partition column chromatography (see Figure 4 of Carpenter and Hayes, 1963). Braces indicate that 1° wedged windows were used to give two schlieren patterns simultaneously. [b] Desalanine-insulin was isolated from an acetone precipitate of an insulin sample which has been subjected to brief treatment with carboxypeptidase. [c] Desamido-insulin was isolated by countercurrent distribution (see Figure 1 of Slobin and Carpenter, 1963b).

TABLE V: Optical Rotatory Properties of Insulin and Derivatives.

Compound	Concn[a] (mg/ml)	pH	$-[\alpha]_{440}$	λ_c
Zinc-free insulin	6.2	7.4	78	258
Zinc-free insulin	4.3	8.0	76	262
Zinc-free insulin	6.2	9.4	77	258
Desalanine-desasparagine-insulin[b]	4.0	8.0	102	242
Desalanine-insulin[c]	5.0	8.0	62	258

[a] All measurements were made in a mixed buffer which was 0.02 M in Tris and 0.025 M in 2-amino-2-methyl-1,3-propanediol. [b] Purified by countercurrent distribution (see fraction A of Figure 5, Slobin and Carpenter, 1963a). [c] Prepared by brief treatment of insulin with carboxypeptidase A and purified by partition column chromatography according to the procedure of Chrambach and Carpenter (1960).

Discussion

Effect of Side Chain. Previous studies on synthetic substrates have led to an outline of the specificity requirements of carboxypeptidase A (for a review see Neurath and Schwert, 1950). One of the most important structural determinants of the substrate is the nature of the carboxyl-terminal residue in the peptide (Neurath, 1960). In general it has been found that, all other things being equal, peptides containing aromatic side chains on the carboxyl-terminal residues are the most susceptible to the action of the enzyme, peptides containing aliphatic side chains are less susceptible, and peptides containing either a positive or negative charge on the side chain are extremely resistant to enzymatic attack.

The model substrates reported here allow a comparison between aliphatic, polar, and negatively charged side chains on the carboxyl-terminal residue. It has been reported that carbobenzoxyglycylglycine is not a substrate for carboxypeptidase (Elkins-Kaufman and Neurath, 1948). Thus, at pH 7.5, replacement of one hydrogen by a methyl group in going from carbobenzoxyglycylglycine to the corresponding alanine derivative increases the rate constant (k) from a value near zero to 1620 M^{-1} min^{-1}. Replacement of the hydrogen in the methyl group of alanine with the polar carboxamide group in going to carbobenzoxyglycylasparagine results in a decrease in the rate constant by a factor of 100. This decrease in the rate constant due to the polar carboxamide group is essentially offset by insertion of an additional methylene group, as is illustrated by the fact that the carbobenzoxyglycyl derivatives of alanine and glutamine have nearly the same k values. Replacement of a hydrogen in the methyl group of alanine with the carboxyl or carboxymethyl groups as in the aspartic acid and glutamic acid derivatives has a profound effect, decreasing the rate constant at pH 7.5 by an estimated 10^5 fold.

Using the carbobenzoxyglycyl derivatives of phenylalanine and tryptophan, Lumry *et al.* (1951, 1955) observed substrate inhibition of carboxypeptidase at substrate concentrations much greater than 0.02 M. However, the corresponding derivative of leucine showed no inhibition at substrate concentrations up to 0.09 M (the highest studied). In the present investigation on the carbobenzoxyglycyl derivatives of alanine, asparagine, and glutamine, at pH values greater than 6.5, the initial velocities were proportional to substrate concentration up to 0.15 M and frequently up to 0.2 M. Such a result indicates a lack of substrate inhibition by these compounds. As a referee has pointed out, this lack of inhibition may well be related to the fact that the K_m values for these substrates are much greater than those for the substrate investigated by Lumry *et al.* (1951).

Comparison of Model Peptides with Insulin. In preliminary work (Slobin and Carpenter, 1963a) we had observed that the rate of release of asparagine from insulin increased with increasing pH above 7.5, whereas the rate of release of aspartic acid from desamidoinsulin decreased under the same conditions. A comparison of the effect of pH on the rate of cleavage of carbobenzoxyglycyl derivatives of asparagine and aspartic acid shows that whereas the rate of release of asparagine has a pH optimum around pH 7.5 the rate of release of aspartic acid has an optimum at pH 6.0 (or below). A similar effect of pH was observed for the carboxypeptidase-catalyzed hydrolysis of carbobenzoxyglycyl derivatives of glutamine and glutamic acid. Thus it appears that the differing effect of pH on the release of asparagine and aspartic acid from insulin and desamidoinsulin, respectively, can be ascribed at least in part to the nature of the side chains on the carboxyl-terminal residue. In this connection Green and Stahmann (1952) reported that carboxypeptidase would hydrolyze polyglutamic acid to a large extent at pH 5.0 but not at all at pH 7.5. The difference in pH–rate profiles for the action of carboxypeptidase on derivatives of aspartic and glutamic acids as compared to their corresponding amides is not unique for this enzyme. A similar observation has been made on papain (Kimmel and Smith, 1954). The pH optimum for the papain cleavage of the neutral peptide, carbobenzoxyglycylglutamic acid diamide, occurred at pH 7.5, whereas the optimum for the cleavage of the acidic compound, carbobenzoxyglycylisoglutamine, occurred at pH 4–5.

Carbobenzoxyglycylalanine is hydrolyzed by carboxypeptidase at about 100 times the rate of corresponding asparagine derivative at all pH values. In insulin the alanine residue is cleaved from 10 to 100 times (depending on pH and concentration) faster than the asparagine residue under the same conditions. Comparison of these two results suggests that the superiority of alanine over asparagine residues in insulin as a substrate for carboxypeptidase is to a large extent a reflection of the relative reactivity of the two residues, as determined on the model peptides. However, this interpretation must be viewed with some caution owing to the fact that the penultimate amino acids of the model peptides (glycine) are not identical with those (cystine and lysine) found in insulin.

Action of Carboxypeptidase on Insulin. Although the insulin–carboxypeptidase system is a relatively simple system with regard to the number of peptide bonds cleaved, it still contains many complexities. Figure 4 portrays a kinetic scheme for the action of carboxypeptidase A on insulin in the monomeric form. In view of the observation that under all conditions alanine is released at least 10 times faster than asparagine, the lower leg of the scheme (Figure 4) involving the sequential release of alanine (reaction 2) followed by asparagine (reaction 4) must be the route of primary significance to the kinetics.

There are four observations which have been made on the action of carboxypeptidase on insulin which are not readily explained in terms of the action of the enzyme on model compounds. (1) In contrast to normal Michaelis–Menten behavior, the rate of release of both alanine and asparagine are increased upon dilution of the insulin from 10 mg/ml to 1 mg/ml. (2) Whereas the pH optimum for the release of alanine and aspara-

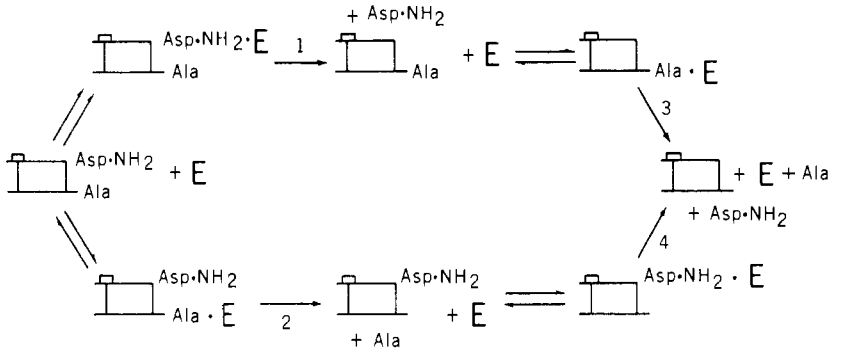

FIGURE 4: A possible kinetic scheme for the action of carboxypeptidase A (E) on the monomer form of insulin.

gine from model peptides is at pH 7.5, the rate of release of both of these groups from insulin increases on going from pH 7.5 to pH 9.0. (3) Although an increase in the ionic strength of the medium up to 0.5 increases the rate of release of amino acids from model peptides (Lumry *et al.*, 1951), the rates of release of alanine and asparagine from insulin are decreased upon increasing the salt concentration. (4) Zinc-free insulin is attacked at a faster rate than zinc insulin (Slobin and Carpenter, 1963a).

The increase in rate of release of alanine and asparagine observed on dilution of the insulin might reflect a release of substrate inhibition of the enzyme by dilution. However, in view of the other observations, it appears more likely that the increase in rate observed upon dilution of the substrate is actually due to an increase in the concentration of the groups accessible to the action of the enzyme. As judged by our own sedimentation studies as well as by those of Fredericq (1956), the following factors decrease the degree of polymerization of insulin in solution: (1) decreasing the concentration of insulin, (2) increasing the pH from 7.5 to 9.0, (3) decreasing the salt concentration, and (4) removing the zinc from the insulin. All of these factors bring about an increase in the rate at which carboxypeptidase releases the carboxyl-terminal groups from insulin. Thus there is a close correlation between those factors which increase the degree of dissociation of insulin polymers and those factors which increase the rate of action of the enzyme on insulin. The evidence suggests that decreases in the degree of aggregation of insulin result in increases in the number of groups accessible to the action of the enzyme. It is possible that some of the changes in conditions which affected the action of the enzyme, such as the pH change, may have brought about a change in the secondary or tertiary structure of a single molecule, resulting in increasing the accessibility of the carboxyl-terminal groups. If such was the case it was not revealed by the optical rotatory dispersion studies which showed a constant λ_c value for insulin between pH 7.4 and 9.4.

The above observations indicate that the kinetic picture proposed in Figure 4 should contain as a minimum a term for the polymeric forms of insulin: $(insulin)_n \rightleftarrows n(insulin)$. The association of insulin in aqueous solution is extremely complex, and there are few definite conclusions that can be reached about this system (Adams, 1962). It is clear, however, that many polymeric forms may exist in solution and that the relative amounts of these aggregates depend quite strongly upon several extrinsic properties of the solution (Steiner, 1952; Fredericq, 1956). This raises the question as to whether any of the polymeric forms of insulin are attacked by the enzyme. If so, equations similar to those depicted in Figure 4 would have to be considered for each polymer of the insulin molecule.

At low insulin concentrations (1 mg/ml) the rate of release of both alanine and asparagine at all pH values followed first-order kinetics for at least 40% reaction if the *monomer* molecular weight was used in the calculations. This fact is an argument for supposing that the monomer is the chief reactive form under conditions of low substrate concentration. On the other hand, at high substrate concentration (10 mg/ml) the release of alanine and asparagine at all pH values followed first-order kinetics only if the *dimer* molecular weight was used in the calculations. This latter observation makes it tempting to propose that the dimer is the principal reactive species at high substrate concentration. In this respect, dilution had a relatively greater effect on the rate of release of asparagine than of alanine. One can conceive of a dimer where the asparagine is buried in dimer formation while the alanine remains more accessible. Upon dissociation to monomers the relative increase in the number of accessible asparagine residues would be several times that of the accessible alanine residues.

The above data indicate that the optimum conditions for the action of the enzyme as derived from studies on model peptides do not necessarily apply to the action of the enzyme on protein substrates. In this particular case the degree of aggregation of the substrate appears to play a dominant role in determining the rate of the carboxypeptidase A catalyzed reaction.

In connection with our results it is interesting to note that Davie *et al.* (1959), in their studies on the action of carboxypeptidase A on β-lactoglobulin, found marked variation in the rate of release of the carboxyl-terminal

amino acids as the pH was increased from 7.6 to 9.2. At pH 7.6 there was a stepwise release of two isoleucine and subsequently two histidine residues. The rate of release of the first equivalent of isoleucine obeyed zero-order kinetics. The stepwise release suggested to the authors that the availability of the two chains of β-lactoglobulin to carboxypeptidase attack was not the same at this pH. At pH 9.2 the two chains were attacked more rapidly than at pH 7.6 and appeared to be equivalent; the release of both isoleucines obeyed first-order kinetics. They attributed these differences in kinetics to a conformational change in the protein substrate. The fact that enzymatic attack on the protein was progressively inhibited by increasing ionic strength suggests that aggregation of β-lactoglobulin may also be of importance. Also, Kassell and Laskowski (1962) found that dilution of a solution of α-chymotrypsin, without changing other conditions, resulted in a large increase in the liberation of amino acids by the action of carboxypeptidase. This result might also be explained by a change in the degree of aggregation of the protein.

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