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The Activation of Bovine Procarboxypeptidase A. I. Isolation and Properties of the Succinylated Enzyme Precursor*

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ABSTRACT: Treatment of bovine pancreatic procarboxypeptidase A with succinic anhydride leads to spontaneous disaggregation into succinylated subunits. By means of gel filtration and chromatography, succinylated subunit I, the immediate precursor of succinylcarboxypeptidase A, has been isolated. The purified zymogen has a sedimentation coefficient of approximately 4 S and a molecular weight of 40,000–42,000.

In comparison to carboxypeptidase A, the zymogen is particularly rich in the dibasic amino acid residues. Tryptic activation results in an enzymatically active species having the properties of succinylcarboxypeptidase.

The study of activation of pancreatic zymogens, notably trypsinogen, chymotrypsinogen, and procarboxypeptidase B, has demonstrated that in each of these systems the formation of an enzymatically active species is dependent on the tryptic cleavage of a unique peptide bond in the amino-terminal region of these proteins (Neurath, 1964). This primary chemical event is believed to be the prerequisite for conformational changes which in turn give rise to the formation of the catalytic site. Analogous studies on the formation of carboxypeptidase A from its inactive precursors have been complicated by the fact that these zymogens exist as tightly bound aggregates of two and three different subunits, respectively (Brown *et al.*, 1961, 1963a), which together make up the protein known as procarboxypeptidase A (Keller *et al.*, 1956, 1958). This physical state of aggregation may be responsible for the fact that *in vitro* the activation of bovine procarboxypeptidase A proceeds very much slower than that of the other pancreatic zymogens (Neurath, 1964).

Disaggregation of procarboxypeptidase into its component subunit fractions can be accomplished by concentrated urea solutions, or in aqueous solutions at pH 10.5 (Brown *et al.*, 1963a). Under the latter conditions, however, the immediate precursor of carboxypeptidase A, "fraction I," becomes denatured and can only be identified by chemical analysis but not by enzymatic function. In the work reported herein, attempts were made to effect disaggregation and separation of the subunits by succinylation of the parent protein, procarboxypeptidase A-S6, with succinic anhydride. It has previously been shown that certain proteins such as hemerythrin (Klotz and Kerestes-Nagy, 1963), porcine heart aspartate transaminase (Polyanovsky, 1965), or rabbit muscle aldolase (Hass, 1964) undergo spontaneous disaggregation under these conditions, presumably through electrostatic repulsion between the negatively charged carboxylate groups which replace the positively charged ϵ-amino groups of lysyl residues. Moreover, Vallee *et al.* (1963) demonstrated that carboxypeptidase A retains its enzymatic function following succinylation and further, that

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tyrosyl residues were succinylated followed by spontaneous desuccinylation (Riordan and Vallee, 1964). The present paper describes the disaggregation of procarboxypeptidase by treatment with succinic anhydride and the isolation and characterization of succinyl fraction I. The mechanism of conversion to the active enzyme is described in the companion paper (Freisheim *et al.*, 1967).

Materials and Methods

Preparation of Procarboxypeptidase A-S6. Procarboxypeptidase A-S6 was isolated from aqueous extracts of acetone powders of bovine pancreas glands and purified by chromatography on DEAE-cellulose according to the procedure described by Yamasaki *et al.* (1963).

Substrates. Hippuryl-*dl*-β-phenyllactic acid (HPLA)[1] was prepared by Dr. W. O. McClure (McClure and Neurath, 1966) or purchased from the Cyclo Chemical Corp., Los Angeles, Calif. Acetyl-L-tyrosine ethyl ester (ATEE) and benzoyl-L-arginine ethyl ester (BAEE) were purchased from the California Corp. for Biochemical Research (Calbiochem, Los Angeles, Calif.).

Reagents and Enzymes. Diisopropylphosphorofluoridate (DFP) was purchased from the Merck Chemical Co. and was diluted to 1 M with anhydrous isopropyl alcohol before use. *Trypsin* was obtained as the salt-free, twice-crystallized, lyophilized product from the Worthington Biochemical Corp. *Bovine carboxypeptidase* A_α was prepared in this laboratory by the method of Cox *et al.* (1964). *1-Fluoro-2,4-dinitrobenzene* (FDNB) was a preparation obtained from Matheson Coleman and Bell and was used without further purification. *Succinic anhydride* was obtained from Eastman Organic Chemicals, Inc. All other chemicals used were of reagent grade.

Protein concentration was determined routinely from measurements of absorbance at 280 mμ. The absorbancy index of purified procarboxypeptidase A-S6 at 280 mμ was assumed to be 1.9 for a 0.1% solution (Keller *et al.*, 1956). The absorbancy index of succinyl fraction I was obtained by relating absorbancy at 280 mμ to protein concentration as determined in the ultracentrifuge using refractometric methods. A refractive index increment of 0.00185 dl g^{-1} at 5500 A was assumed (Doty and Edsall, 1951).

Column Chromatography and Gel Filtration. DEAE-cellulose (0.84 mequiv/gm) was a product of the Carl Schleicher & Schuell Co. The adsorbent was washed with 0.10 N potassium hydroxide followed by several changes of distilled water at room temperature. The pH was then adjusted to 4.5 with concentrated phosphoric acid and the suspension was deaerated to remove any bicarbonate or trapped air. The pH was adjusted to 8.0 with potassium hydroxide and the exchanger was equilibrated with 0.005 M potassium phosphate buffer prior to the chromatography of procarboxypeptidase. Hydroxylapatite was obtained as a suspension in 0.001 M sodium phosphate buffer (pH 6.5) under the trade name of Bio Gel-HT from Bio-Rad Laboratories. The adsorbent was washed five times with four volumes of 0.005 M potassium phosphate buffer (pH 6.5) and decanted to remove the very fine particles. Serva hydroxylapatite cellulose (Gallard-Schlesinger Chemical Manufacturing Co.) was admixed with Bio Gel-HT (approximately 3:1, v/v). This mixture will be referred to as hydroxylapatite cellulose. Sephadex G-100 was packed into a column in the cold after equilibration for 72 hr at room temperature with buffer as recommended by Pharmacia (Uppsala, Sweden).

Assay Procedures. Esterase activities were determined by titration with a glass electrode on the TTT-1 Radiometer autotitrator equipped with an Ole Dich recorder (Copenhagen) at 25° with 0.090 to 0.10 N standardized sodium hydroxide. BAEE (0.01 M in 0.01 M Tris–0.05 M $CaCl_2$–0.10 M KCl (pH 7.8)) was employed to assay for tryptic activity. HPLA (0.01 M of the DL-sodium salt in 0.005 M sodium Veronal–0.05 M NaCl (pH 7.5)) was used as a substrate for carboxypeptidase A activity. ATEE (0.01 M in 0.01 M Tris–0.10 M KCl (pH 8.0)) was used to assay for *chymotrypsin-like activity*. Relative esterase activity units are expressed as the uptake of microequivalents of base per minute per milliliter of protein solution. Specific esterase activities are expressed in terms of K_{obsd}^0 which has the units of microequivalents of base consumed per minute per microgram of protein. "Potential" activity refers to the enzymatic activity which can be elicited from a zymogen under stated conditions of activation.

Amino-terminal analyses were carried out using the FDNB method of Sanger (1945) as modified by Fraenkel-Conrat *et al.* (1955).

Sedimentation analysis was carried out in a Beckman Model E analytical ultracentrifuge equipped with phase-plate Schlieren optics. The analyses were performed in either a single- or double-sector synthetic boundary cell at 59,780 rpm. Plate negatives of the observed Schlieren patterns were taken at regular time intervals during the run and the radial boundary position of the sedimenting protein was measured on a microcomparator at each time interval. The sedimentation constants were calculated from least-squares slopes of a plot of the log of the radial boundary position *vs.* time (Schachman, 1957). Viscosities were measured at 20.00 ± 0.01° and correction for buffer density was obtained by pycnometry at 20.0°.

Sedimentation Equilibrium Analysis. Sedimentation equilibrium measurements at 3–5° were performed in the ultracentrifuge employing Rayleigh interference optics as described by Richards and Schachman (1959) at speeds ranging from 5770 to 9946 rpm. A double-sector synthetic boundary cell was employed and the protein sample to be analyzed was layered over FC 43 (3M Chemicals) at column heights of 2.0–2.5 mm. Hinge-point shift was followed in serial pat-

[1] The following abbreviations are used: HPLA, hippuryl-DL-β-phenyllactic acid; ATEE, acetyl-L-tyrosine ethyl ester; BAEE, benzoyl-L-arginine ethyl ester; FDNB, 1-fluoro-2,4-dinitrobenzene; PCP, procarboxypeptidase.

terns; equilibrium was assumed to be attained when no further shift of fringes could be detected. The data were plotted as in the ln C *vs.* x^2 (when C = fringe number; x = distance from the axis of rotation), with extrapolation to the menisci. The apparent weight-average molecular weight over the mass of the cell was evaluated from the total change in concentration across the cell relative to the initial concentration according to the equation of Lansing and Kramer (1935). The weight-average molecular weight over the volume of the cell was evaluated from the equation described by Adams (1964).

Amino Acid Analysis. Amino acid analyses were performed according to Spackman *et al.* (1958) on the Beckman amino acid analyzer, Model 120. Acid hydrolysis was carried out with constant boiling HCl (5.7 N) *in vacuo* at 108° in an oven. The samples were frozen in solid CO_2–2-methoxyethanol and evacuated by means of a mechanical oil pump for about 5 min before sealing. The samples were hydrolyzed for periods of 24, 49, 72, and 109 hr. The hydrolysates were taken to dryness by evaporation under reduced pressure by means of a mechanical pump at 40°. The tubes were allowed to evacuate for 40 min to ensure thorough removal of HCl vapors. The dried samples were taken up in 0.2 N sodium citrate buffer (pH 2.2) containing a mixture of L-2-amino-3-guanidopropionate and β-thienylalanine as internal standards to correct for slight variabilities between the long- and short-column determinations as suggested by Walsh and Brown (1962). Cysteine and methionine were analyzed as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid (Moore, 1963). Tryptophan determinations were carried out according to Bencze and Schmid (1957) in 0.10 N NaOH from measurements of ultraviolet absorption spectra. Hydroxyamino acid content was obtained by extrapolation to zero time of hydrolysis while other amino acids were calculated by extrapolation to maximum recovery.

Spectrophotometric titrations of carboxypeptidase A_α in 1.0 M NaCl and of succinyl fraction I in water at 295 mμ were performed between pH 6.25 and 13.0 by the addition of small amounts of standardized NaOH solutions to the protein solutions. The titrations were carried out at 25° in a pH-Stat (Radiometer, Copenhagen); volume corrections were made assuming volume additivity. Absorbance measurements were made in a Zeiss PMQ II spectrophotometer. Protein concentrations were determined spectrophotometrically and molar absorbancies at 295 mμ were calculated assuming molecular weights of 34,600 for carboxypeptidase A_α (Bargetzi *et al.*, 1963) and 41,000 for succinyl fraction I (*vide infra*). The reversibility of the titrations was checked by the addition of appropriate amounts of standardized HCl solutions to the protein solutions at high pH. These titrations were not reversible above pH 11. Owing to the time-dependent ionization of phenolic groups at pH values of 10–13 (Tanford and Roberts, 1952), absorbance measurements were performed within 2 min after the addition of base. The change in molar absorbancy for the ionization of free L-tyrosine in 1.0 M NaCl at 295 mμ is reported to be 2350 (Fujioka and Imahori, 1963). The molar absorbancy for the complete ionization of free L-tyrosine in water at 295 mμ is 2300 (Tanford, 1950). These values were used to calculate the number of ionized tyrosyl residues.

TABLE I: The Succinylation of PCP-S6 with Succinic Anhydride.[a]

Wt Ratio of Succinic Anhydride: PCP-S6 (x:1.0)	Esterase Act.[b]	Sedimentation Velocity[c]	
		s_{20}	Protein Concn (mg/ml)
x = 0.11	5.4	5.03[d]	10.0
x = 0.24	14.0	4.66 + 3.44	9.7
x = 0.41	25.9	4.43 + 2.44	9.7
x = 0.64	32.4	3.11	9.7
x = 1.00	32.2	2.92	10.0
x = 1.50	21.6	2.35	10.0
x = 2.24	5.9	1.39	10.0
x = 3.19	2.5	1.00	11.2
x = 4.00	1.8	1.47	13.2

[a] Succinylated preparations of procarboxypeptidase were dialyzed for 48 hr against 500 ml of 0.0125 M Tris-chloride–0.05 M KCl (pH 8.0) prior to sedimentation analysis and tryptic activation. [b] After 15-min activation with trypsin at 25° (trypsin:procarboxypeptidase weight ratio, 1:30). Esterase activities (HPLA as substrate) are expressed as μequiv of OH uptake min^{-1}, μg of PCP-A-S6 × 10^3. The maximum carboxypeptidase content can be calculated to correspond to a specific activity of 83 in the units used here ((35,000/88,000) × 0.208 × 10^3). [c] The material appeared monodisperse except where noted. [d] Additional slower sedimenting material was not in sufficient concentration to measure accurately.

Selection of Optimum Conditions of Disaggregation. The effects of succinylation with succinic anhydride were followed by sedimentation analysis in the ultracentrifuge. In these experiments approximately 30 mg of procarboxypeptidase was dissolved in 3 ml of distilled water containing 10^{-5} M DFP. Succinic anhydride was added in increasing proportions to each of nine aliquots. The pH was maintained between 7.9 and 8.0 during succinylation in a pH-Stat by the addition of 6 N KOH dispensed from a microsyringe. Depending on the amount of succinic anhydride used, the reaction was complete within 5–30 min. The succinylated preparations were dialyzed for 48 hr against 500 ml of 0.0125 M Tris-chloride–0.05 M KCl (pH 8.0) to remove any labile *O*-succinyl groups (Vallee *et al.*, 1963). Esterase activities were measured with HPLA after activation with trypsin for 15 min at 25° (trypsin:procarboxypeptidase weight ratio, 1:30).

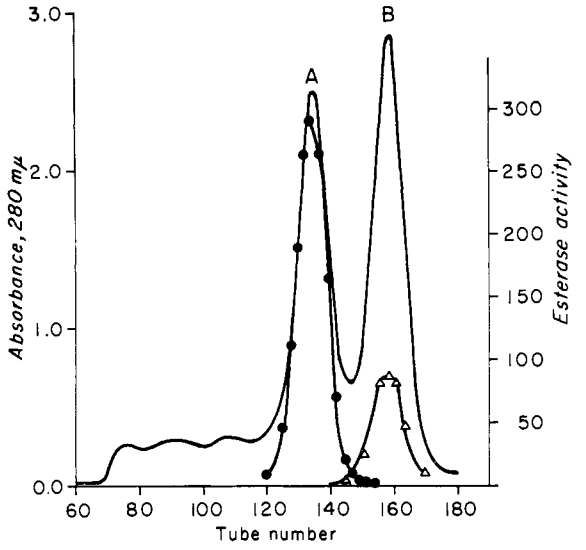

FIGURE 1: Sephadex G-100 effluent profile of succinylated PCP-S6. The details are given in the text. (———) Absorbancy at 280 mμ. Esterase activities are expressed as microequivalents of OH taken up min^{-1} ml^{-1} of protein solution. HPLAse (●—●) and ATEEase (△—△).

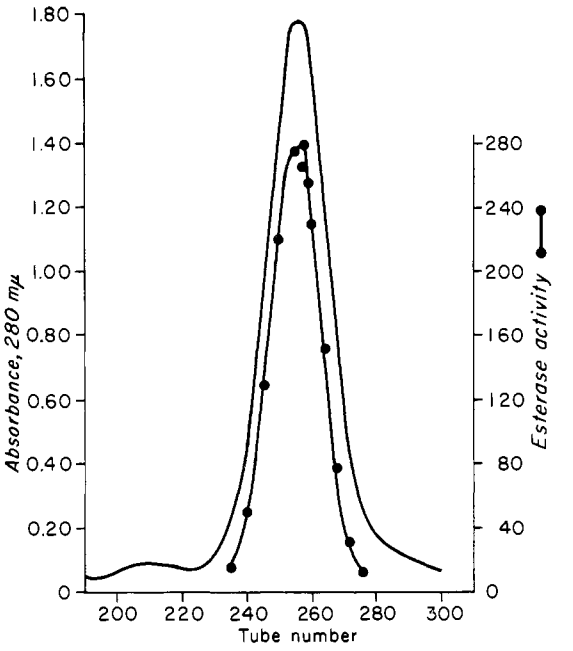

FIGURE 2: Sephadex G-100 gel filtration effluent profile of succinyl fraction I (peak A). The column dimensions were 4.5 × 120 cm; the elution buffer was 0.025 M Tris–0.50 M KCl–1.0 M urea (pH 8.0). HPLA esterase activities are expressed as microequivalents of OH min^{-1} ml^{-1} of protein solution (●—●). Absorbance at 280 mμ (———).

Results

Disaggregation of Procarboxypeptidase by Succinylation. The effects of succinylation on the sedimentation behavior of procarboxypeptidase are shown in Table I. Also shown are the changes in esterase activity measured after 15 min of tryptic activation at 25°. These conditions are sufficient to complete the activation of succinylated procarboxypeptidase, whereas native procarboxypeptidase A-S6 requires 4 hr of activation at 36° by three times as much trypsin (Yamasaki *et al.*, 1963). The highest potential esterase activity recorded after tryptic activation in Table I is less than 40% of that expected from complete activation of the native zymogen; this value could not be increased by more extended tryptic activation but, on further purification, increased to 125–141%. In contrast, the lower esterase activities of less extremely succinylated material reflect the slow activation characteristic of unmodified procarboxypeptidase.

Under succinylation conditions which evoke maximum esterase activity, the protein appears to be monodisperse with a sedimentation coefficient of approximately 3 S, a value characteristic of carboxypeptidase A. Since at weight ratios greater than 1.0 the product of activation contained progressively less activity, the effects of succinylation in this region on sedimentation behavior have not been further explored. All of the succinylated protein described elsewhere in this report was prepared with a protein:succinic anhydride weight ratio of 1.0.

The degree of modification of the free amino groups of succinylated procarboxypeptidase and its unmodified counterpart was determined by means of the ninhydrin reaction (Moore and Stein, 1948) using phenylalanine as a reference standard. The succinylated procarboxypeptidase contained 10.5 ± 1.0 free amino groups, in contrast to 31.2 ± 1.0 for procarboxypeptidase and 14.9 ± 0.1 for carboxypeptidase A_γ. These data for the native proteins are in good agreement with the known content of 16 amino groups in carboxypeptidase A_γ (Bargetzi *et al.*, 1963) and of 31 amino groups in procarboxypeptidase (Yamasaki *et al.*, 1963). Hence approximately two-thirds of the lysyl residues of procarboxypeptidase have been succinylated.

Isolation and Purification of Succinyl Fraction I. In a typical experiment approximately 370 mg of procarboxypeptidase in 40 ml of distilled water containing 10^{-5} M DFP was succinylated with an anhydride:protein ratio of 1.0. The succinylated zymogen was then dialyzed for 24 hr against 4 l. of 5 × 10^{-4} M Tris-HCl–10^{-2} M KCl (pH 8.0) and subsequently lyophilized. The lyophilized material was dissolved in 7 ml of 0.025 M Tris-HCl–0.50 M KCl (pH 8.0) containing 4% sucrose. The use of sucrose aids in a more uniform sample application to a gel filtration column and thereby increases the resolution of the protein species in the effluent. The succinylated procarboxypeptidase solution was layered onto a 4.5 × 130 cm Sephadex G-100 column which had been previously equilibrated with 0.025 M Tris-HCl–0.50 M

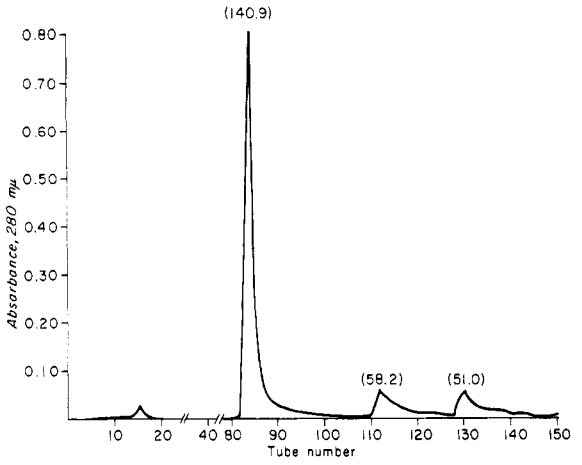

FIGURE 3: Hydroxylapatite cellulose chromatography of succinyl fraction I. The HPLA esterase activities shown in parentheses are expressed as per cent of native carboxypeptidase A.

Stepwise Elution System	
Potassium Phosphate Concn (M) (pH 6.5)	Effluent Fractions 1-20 (sample applied)
0.005	21–38
0.015	39–59
0.030	60–80
0.10	81–110
0.25	111–128
0.50	129–150

KCl–1.0 M urea (pH 8.0). The use of urea was found to give increased resolution of the protein species appearing in the effluent and at this concentration had no adverse effect on potential carboxypeptidase activity. The effluent fractions resulting from gel filtration on Sephadex G-100 were tested for potential exopeptidase and endopeptidase activities toward HPLA and ATEE, respectively, with the results shown in Figure 1. These two activities, characteristic of carboxypeptidase and fraction II (of procarboxypeptidase), respectively, were well separated from each other, indicating a considerable difference in molecular weight of the two proteins.

A second filtration of succinyl fraction I on a 4.5 × 130 cm Sephadex G-100 column employing the same buffer system resulted in an effluent profile (Figure 2) which suggested a reasonable homogeneity of the product.

Further purification of succinyl fraction I was achieved by chromatography on a hydroxylapatite cellulose column (1.9 × 32 cm). The mixture of hydroxylapatite with hydroxylapatite cellulose was found to give flow rates of the order of 20–30 ml/hr. The column was first equilibrated with 0.005 M potassium phosphate (pH 6.5), then a stepwise elution system was employed as indicated in Figure 3. As shown in Figure 3, most of the protein material emerging from the column appeared as a single peak following elution with 0.10 M potassium phosphate (pH 6.5).

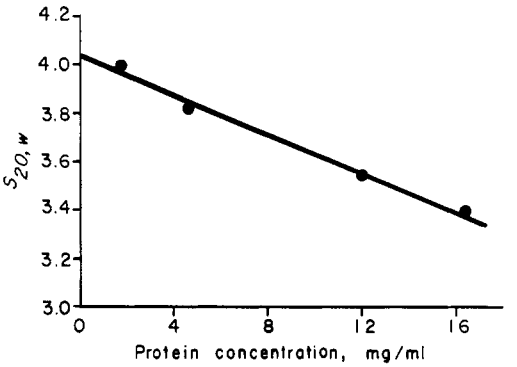

FIGURE 4: Dependence of the sedimentation velocity of succinyl fraction I upon protein concentration. The buffer used for these studies was Γ/2 − 0.10 potassium phosphate (pH 6.5). See text for further details.

Table II gives a typical scheme for the purification of succinyl fraction I according to the procedure just described. After the second gel filtration only the material in the peak which exhibited constant potential specific activity was pooled (*ca.* 22% of the total protein).

Properties of Succinyl Fraction I. The characterization of succinyl fraction I was carried out on the fraction of highest potential activity, obtained from the hydroxylapatite cellulose column.

Sedimentation Analysis. A study of sedimentation velocity in potassium phosphate buffer (pH 6.5), ionic strength 0.10, at 3–6° indicated a decrease of $s_{20,w}$ with increasing protein concentration (Figure 4).

TABLE II: A Typical Scheme for the Purification of Succinyl Fraction I from Succinylated Procarboxypeptidase A-S6.

Stage	Yield (mg of protein)	Potential HPLase Sp Act.[a]	% Recov[b]
Succinylated PCP-S6	370	32	
First gel filtration	109	193	100
Second gel filtration	24	220	22
Hydroxylapatite cellulose chromatography	17	293	16

[a] Potential specific activity units (after tryptic activation) are given as μequiv of OH consumed min^{-1} μg^{-1} of protein × 10^3. [b] Per cent recoveries are based on the amount of succinyl fraction I isolated from the first gel filtration.

TABLE III: Molecular Weights Determined from Sedimentation Equilibrium Analysis.

Protein (mg/ml)	pH	Buffer	M_w[a]	M_v[a]
1.7	6.5	PO_4(K) $\Gamma/2 = 0.1$	43,800	41,500
4.6	Same	Same	42,200	38,100
4.6	Same	Same	43,000	39,400
4.6	Same	Same	43,800	40,000
6.4	Same	Same	41,300	41,300
5.3	8.0	0.0125 M Tris–0.25 M KCl	40,000	38,200
			42,350 ± 1,500	39,750 ± 1,470

[a] Apparent weight-average molecular weight over the mass of the cell (M_w) and over the volume of the cell (M_v).

Extrapolation to zero protein concentration gave a value of $s_{20,w} = 4.04$ S.

Sedimentation Equilibrium and Molecular Weight. The results of these calculations are summarized in Table III. The partial specific volume used in these calculations was assumed to be 0.73 ml/g; the apparent weight-average molecular weight was approximately 39,800–42,400.

Absorbancy Index. An absorbancy index of 18.3 for a 1% solution at 280 mμ was determined by relating absorbance to protein concentration as measured in the ultracentrifuge employing refractometric methods. The refractive index gradient was assumed to be 0.00185 dl g^{-1} (*vide supra*).

Degree of Succinylation. The number of ninhydrin-titratable free amino groups was found to be 4.2 ± 0.2 using phenylalanine as a reference standard. Riordan and Vallee (1964) have similarly observed that reaction of carboxypeptidase with a 96-fold molar excess of succinic anhydride was accompanied by a decrease in the number of free amino groups from 15 to *ca.* 4 based on ninhydrin titration.

The degree of acetylation of the phenolic hydroxyls of tyrosyl residues has been reported by Simpson *et al.* (1963) for acetylcarboxypeptidase and more recently by Riordan *et al.* (1965) and Perlmann (1966) for acetylated pepsin and pepsinogen. These investigators have shown that incubation of the acetylated proteins with 0.01 M hydroxylamine (pH 7.5, 23°) resulted in increased molar absorptivities at 278 mμ. The number of tyrosines acetylated was determined from an increase in absorptivity at 278 mμ based on a molar absorptivity of 1160 for the transition of *N,O*-diacetyl- to *N*-acetyltyrosine (Simpson *et al.*, 1963). The number of phenolic hydroxyls of tyrosine modified in succinyl fraction I was estimated to be 6.5 using this method (Table IV).

Amino Acid Composition. The results, based on internal standards, are summarized in Table V. The apparent number of lysyl residues increased by one after 109 hr of hydrolysis based on the number of micromoles of amino acid recovered. Gounaris and Ottesen (1965) in their studies of succinylated subtilopeptidase have suggested that this time-dependent increase in the number of free lysines is the result of a slow hydrolysis of the ε-*N*-succinimide derivative of lysine. The minimum molecular weight, calculated from the data in Table V, is 42,106 which compares well with the values obtained by sedimentation equilibrium analysis (Table III).

Spectrophotometric Titration of the Tyrosyl Residues of Carboxypeptidase A_α and Succinyl Fraction I. The finding of 17 tyrosyl residues for succinyl fraction I is not in accord with the data of Bargetzi *et al.* (1963) for carboxypeptidase A_α and A_γ which have been demonstrated to contain 19 such residues.

To determine the total number of tyrosyl residues of succinyl fraction I by a second procedure, spectrophotometric titrations were performed. For purposes of comparison, titrations were also performed on carboxypeptidase A_α, since these results had not been previously reported.

The titration curve for carboxypeptidase A_α at 295 mμ (25°) is indicated in Figure 5. Between pH 8 and 10.5 the increase in molar absorptivity of carboxy-

TABLE IV: Deacylation of *O*-Succinyltyrosine Residues in Succinyl Fraction I.

Incubn Time (min)[a]	Δa_M^{278} [b]	Number of *O*-Succinyltyrosine Residues Deacylated[c]
780	3572	3.1
850	4074	3.5
1780	7520	6.5

[a] Incubation of succinyl fraction I (0.65 mg/ml) in 0.01 M Tris–0.01 M NH_2OH (pH 7.5) at 25°. [b] No change in the molar absorptivity increment (Δa_M^{278}) was observed for a succinyl fraction I solution (0.65 mg/ml) in 0.01 M Tris (pH 7.5) over the same time interval. [c] $\Delta a_M^{278}/1160$ = number of tyrosines deacylated.

TABLE V: Amino Acid Composition of Succinyl Fraction I.

	Residues/ 10^5 g of Protein	g of Residue/ 10^5 g of Protein	Min Mol Wt	Residues/ 40,000 g of Protein	Nearest Integer/ 40,000 g	Nearest Integer × Min Mol Wt	Integral No. × Mol Wt of Residue
Lysine	42.0	5,388	2,378	16.8	17	40,426	2,179
Histidine	23.3	2,559	4,287	9.3	9	38,583	1,235
Arginine	35.3	5,476	2,850	14.0	14	39,900	2,187
Aspartic acid	82.7	9,515	1,209	33.1	33	39,879	3,798
Threonine	63.0	6,364	1,587	25.2	25	39,675	2,527
Serine	76.5	6,662	1,306	30.6	31	40,486	2,700
Glutamic acid	100.4	12,962	995	40.2	40	39,812	5,164
Proline	38.2	3,714	2,612	15.3	15	39,180	1,457
Glycine	66.5	3,799	1,502	26.6	27	40,554	1,542
Alanine	60.2	4,277	1,661	24.1	24	39,864	1,706
Valine	55.0	5,454	1,818	22.0	22	39,996	2,182
Isoleucine	54.6	6,178	1,831	21.8	22	40,282	2,490
Leucine	74.7	8,456	1,338	29.9	30	40,140	3,396
Tyrosine	42.4	6,925	2,335	17.0	17	40,035	2,774
Phenylalanine	45.0	6,627	2,220	18.0	18	39,960	2,650
Half-cystine[a]	4.84	499		1.94	2		204
Methionine[a]	9.96	1,307		3.98	4		525
Tryptophan[b]	20.2	3,765		8.1	8		1,490
		99,927			350	39,919 ± 506	40,206
							1,900[c]
							42,106

[a] Determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation (Moore, 1963). [b] Determined spectrophotometrically according to Bencze and Schmid (1957). [c] Addition of the molecular weight of 19 succinyl groups, assuming that 13 lysines and 6 tyrosines have been succinylated.

peptidase A_α was 18,500, indicating an ionization of 7.9 tyrosyl residues. This value agrees with the data reported by Fujioka and Imahori (1963) for carboxypeptidase A_γ in this pH range. From pH 10.5 to 12 a further increase in molar absorptivity of 26,500 was observed, corresponding to the ionization of 11.3 tyrosyl residues, a value approximately 3 residues higher than that of 8.5 residues reported by these workers in this pH range. The total increase in molar absorptivity at 295 mμ (between pH 8 and 12) of 45,000 for carboxypeptidase A_α corresponds to the ionization of 19.2 tyrosyl residues per mole of protein. This value agrees with the value of 19 determined by amino acid analysis (Bargetzi *et al.*, 1963).

The titration curve for succinyl fraction I at 295 mμ and at 25° is shown in Figure 6. The shape of the titration curve differs significantly from that observed for carboxypeptidase A_α. As the pH was progressively increased a slight inflection point was observed at approximately pH 10.9. In contrast to the titration curve for carboxypeptidase A_α, the molar absorptivity curve for succinyl fraction I begins to plateau at pH 12, but then progressively increases further up to pH 13, indicating a definite stage of ionization of tyrosyl residues. The total increase in molar absorptivity at 295 mμ for succinyl fraction I between pH 8 and 13 corresponds to an ionization of 19.9 residues of tyrosine per mole of protein. This value is one residue higher than that reported for carboxypeptidase A_α (*vide supra*) and three residues higher than the value of 17 determined by amino acid analysis (Table V).

Amino-Terminal Analysis. Although the procedure was repeated on several preparations of succinyl fraction I, no amino-terminal amino acid residue could be detected following reaction with FDNB. This negative finding may be due to succinylation of the α-amino group during the reaction of procarboxypeptidase with succinic anhydride.

Enzymatic Properties. Following tryptic activation, succinyl fraction I displayed specific esterase activities of $k_{obsd}^0 = 0.229$–0.270. Bargetzi *et al.* (1963) report a value of $k_{obsd}^0 = 0.208$ for native carboxypeptidase A_α. The increase in enzymatic activity to 110–130% of that of native carboxypeptidase is in agreement with the value of 125% reported by Riordan and Vallee (1964) for succinylcarboxypeptidase. The mechanism of activation of succinyl fraction I will be reported

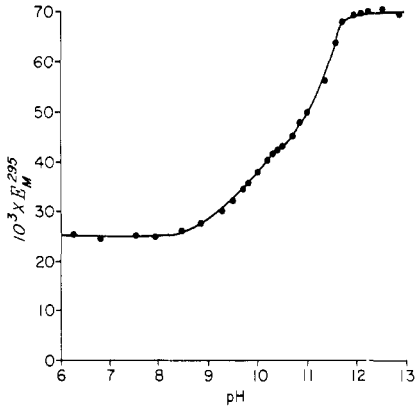

FIGURE 5: Spectrophotometric titration curve of carboxypeptidase A_α at 295 mμ in 1.0 M sodium chloride at 25°. The details are given in the text.

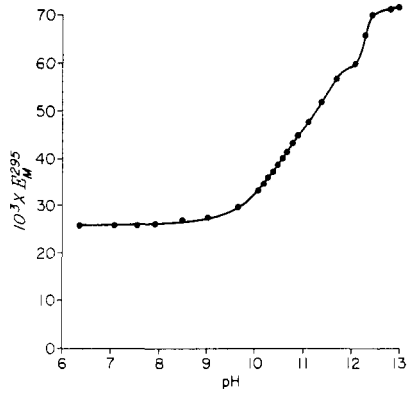

FIGURE 6: Spectrophotometric titration curve of succinyl fraction I at 295 mμ in water at 25°. The details are described in the text.

in the accompanying paper (Freisheim *et al.*, 1967).

Discussion

A detailed investigation of the mechanism of formation of bovine pancreatic carboxypeptidase A from its inactive precursor requires the separation of subunit I from the other components of the aggregate known as procarboxypeptidase A-S6. The only effective procedure previously known required exposure of the aggregate for 24 hr at room temperature to glycine–LiCl solutions at pH 10.5. Under these conditions, a fraction precipitated which resembled carboxypeptidase A_α in amino acid composition (Table VI) but which was denatured and hence functionally inactive (Brown *et al.*, 1963a).

The methods of disaggregation by succinylation previously employed with other systems (Klotz and Kerestes-Nagy, 1963; Polyanovsky, 1965; Hass, 1964) appeared as a promising approach, particularly since Vallee *et al.* (1963) and Riordan and Vallee (1964) demonstrated that succinylated carboxypeptidase A is enzymatically active. The procedures of gel filtration and of chromatography on hydroxylapatite cellulose have proved to be effective in separating the succinylated products of disaggregation from each other. Hydroxylapatite has been previously used by other investigators for the chromatography of acidic proteins (Levin, 1962) and, as applied to the present system, resulted in the separation of derivatives differing from each other in potential enzymatic activity and probably also in degrees of succinylation (Figure 3). The specific conditions applied here for the isolation of succinyl fraction I have been chosen so as to yield maximal potential enzymatic activity. Furthermore, since the conditions of the succinylation were chosen to be optimal in terms of subsequent activability, rather than exhaustive in terms of complete succinylation, it is probable that a family of proteins was produced which differ in their extent and positions of succinylation. These variations are suggested by the data in Figure 3, where the products of activation in each of several peaks from hydroxylapatite columns differ in their specific activity. Nevertheless, the apparently homogeneous behavior of the purified succinyl fraction I in the ultracentrifuge (Figure 4) and during Sephadex chromatography (Figure 2) do indicate that the protein

TABLE VI: Comparison of the Amino Acid Compositions of Inactive Fraction I and Carboxypeptidase A_α with That of Succinyl Fraction I.

	Residues/Mole of Protein		
	Succinyl Fraction I	Inactive Fraction I[a]	Carboxypeptidase A_α[b]
Lysine	17	15	15
Histidine	9	7	8
Arginine	14	12	11
Aspartic acid	33	27	28
Threonine	25[c]	24	28 (25)[c]
Serine	31[c]	33	33 (31)[c]
Glutamic acid	40	28	25
Proline	15	11	10
Glycine	27	25	23
Alanine	24	20	20
Half-cystine	2	2.5	2
Valine	22	16	16
Methionine	4	3	3
Isoleucine	22	22	20
Leucine	30	24	23
Tyrosine	20	18	19
Phenylalanine	18	16	16
Tryptophan	8	8	8

[a] From the data of Brown *et al.* (1963a). [b] From the data of Bargetzi *et al.* (1963). [c] Based on a linear extrapolation to 0 hr of hydrolysis.

moiety of these preparations was essentially homogeneous, whereas the number and distribution of the negative succinyl charges may well vary. It should be emphasized that no attempt was made to arrive at a single and unique species of modified zymogen. Rather, an arbitrary set of conditions was selected which yielded good disaggregation without major loss in activability.

Activity measurements of the activated fractions have enabled the location of certain but not all of the components originally present in procarboxypeptidase A-S6. Thus besides succinyl fraction I, succinylated fraction II and residual undissociated succinyl procarboxypeptidase could be readily identified by use of acetyltyrosine ethyl ester as substrate. However, fraction III would be more difficult to locate because of lack of any demonstrable enzymatic activity (Brown *et al.*, 1961). Indeed, when the same procedures were applied to the succinylation and chromatographic separation of procarboxypeptidases A-S6 and -S5 (Brown *et al.*, 1963b), respectively, no qualitative differences in the chromatograms could be seen; however, the recoveries of the components from procarboxypeptidase A-S5 were complete whereas in the case of -S6, a yield of only approximately 70% could be obtained.

Purified succinyl fraction I appeared to be homogeneous in the ultracentrifuge and to be larger than carboxypeptidase A (Table VI). All amino acid residues in succinylated fraction I were found to be at a level equal to or greater than the corresponding level in carboxypeptidase A_α. The only exception is tyrosine (*vide infra*). Thus the size and composition of succinylated fraction I are consistent with its precursor role for carboxypeptidase. In contrast, the inactive "fraction I" isolated by Brown *et al.* (1963a) from a pH 10.5 disaggregation system has an amino acid composition almost identical with that of carboxypeptidase A_α. Hence that denatured material must have already undergone limited proteolysis similar to that accompanying normal activation.

In succinyl fraction I, "half-cystine" (determined as cysteic acid) and tryptophan, and possibly also threonine and serine, occur in the same amount in the succinylated precursor as in carboxypeptidase A_α. As judged by ninhydrin analysis, 13 of the 17 lysine residues/molecule had become succinylated. The value of four free ε-amino groups in the zymogen compares favorably with the same number of free groups found by Riordan and Vallee (1964) for succinylcarboxypeptidase. In contrast to succinylcarboxypeptidase, however, the succinylated precursor contained *O*-succinyltyrosine residues as indicated by deacylation with neutral hydroxylamine. Moreover, a total of 17 tyrosyl residues/molecule was obtained by amino acid analysis, whereas spectrophotometric titrations at 295 mμ indicated a total of 20 tyrosyl residues. The increase in molar absorbancy of 13,554 at 295 mμ between pH 12 and 13 (Figure 6) corresponds to the ionization of about six phenolic groups of tyrosine. Since six to seven *O*-succinyltyrosine residues were deacylated by treatment with hydroxylamine (Table IV), this portion of the titration curve may reflect the deacylation of these residues. It is assumed that the *O*-succinyltyrosine was not completely converted to free tyrosine during acid hydrolysis, and the higher values of tyrosine content taken from the spectrophotometric titrations are, therefore, believed to be the more reliable estimate of total tyrosine.

As judged by end group analysis, the α-amino group of the zymogen has become succinylated as well as the lysine and tyrosine residues; thus no amino-terminal residue could be detected under any circumstances following reactions with FDNB, whereas native procarboxypeptidase A-S6 yielded N-terminal lysine, aspartic acid, and half-cystine (Brown *et al.*, 1963a).

In contrast to native procarboxypeptidase A-S6, succinyl fraction I is rapidly activated by trypsin, and also by certain other proteolytic enzymes. Moreover, fraction II is not an obligatory participant in the activation mechanism (Freisheim *et al.*, 1967). The enhancement of the rate of tryptic activation is probably the result of prior disaggregation and suggests a functional relation of the activation site to the sites involved in subunit interaction. These and other questions relating to the molecular architecture of the aggregate will be considered in greater detail in the companion paper.

The observation that procarboxypeptidase A-S6 can be disaggregated by succinylation would appear to confirm that covalent linkages cannot participate in maintaining the quaternary structure of the molecule. It is more likely that electrostatic, hydrogen, or hydrophobic bonds are operative at the binding sites between the subunits. In further support, there is accumulating evidence that fraction II, the potential endopeptidase, forms a complex with carboxypeptidase A_α (Brown *et al.* (1963b) and W. D. Behnke, personal communication). The latter observations further indicate that the groups involved in the interaction between the subunits must be largely intact in active carboxypeptidase.

The isolation of the subunit precursor of carboxypeptidase A from the native quaternary structure of the zymogen provides the possibility of examining the nature of the primary activation mechanism without the complications of aggregate structure. Although it is true that the subunit is isolated in a chemically modified form, it has the expected size and composition of a subunit precursor of carboxypeptidase and is fully and rapidly converted to an enzyme having the characteristics of succinyl carboxypeptidase A. Some of the details of this activation process are the subject of the following communication.

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