Procarboxypeptidase A-S6.* Further Studies of its Isolation and Properties

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A chromatographic procedure for the isolation of procarboxypeptidase A-S6 from aqueous extracts of acetone powders of bovine pancreas glands has been described. The purified zymogen is similar to that previously described by Keller *et al.* (1956, 1958), and has a sedimentation coefficient of 6 Svedberg units, in contrast to another form of bovine pancreatic procarboxypeptidase which has a sedimentation coefficient of 5 Svedberg units (Brown *et al.*, 1963b). Procarboxypeptidase A-S6 has also been characterized by complete amino acid analysis, and by amino terminal analysis, the latter yielding half-cystine, lysine, and aspartic acid (or asparagine) in stoichiometrically significant quantities. It is concluded from these data that procarboxypeptidase A-S6 is composed of three polypeptide chains. The action of carboxypeptidase A on the zymogen gives rise to several amino acids, notably leucine, tyrosine, valine, and phenylalanine. Measurements of the sedimentation coefficient, partial specific volume, and sedimentation equilibrium indicate 87,000 as the most probable value for the molecular weight.

Keller *et al.* (1956, 1958) have described the isolation in pure form of procarboxypeptidase A from aqueous extracts of acetone powders of bovine pancreas glands. The purified zymogen was reported to have a molecular weight of approximately 95,000. Upon activation, the proenzyme gave rise first to an endopeptidase which hydrolyzes the substrate acetyl-L-tyrosine ethyl ester and then to carboxypeptidase A. The most highly purified preparations obtained by Keller *et al.* (1958) were isolated by a procedure which included fractionation with ammonium sulfate and chromatography on DEAE-cellulose. Renewed attention was focused on this zymogen when it was found that it is composed of subunits (Brown *et al.*, 1961) and that the endopeptidase and carboxypeptidase A activities are associated with two different subunits of the protein. The more recent finding that procarboxypeptidase in fact exists in two states of molecular aggregation, characterized by sedimentation coefficients of 6 and 5 Svedberg units, respectively (Brown *et al.*, 1963a,b), has emphasized the need to reinvestigate the chromatographic behavior of this zymogen, and to characterize more fully, by chemical and enzymatic means, the properties of the two forms of the zymogen. The present report describes additional studies on the isolation and chemical characterization of procarboxypeptidase A-S6, the zymogen previously described by Keller *et al.* (1956, 1958).

MATERIALS AND METHODS

Acetone Powders.—Acetone powders of bovine pancreas glands were either prepared locally by the methods of Fischer and Stein (1954) and Keller *et al.* (1956), or were supplied by Worthington Biochemical Corporation and by Eli Lilly and Co.[1] Only powders were used that yielded minimal amounts of active carboxypeptidase and trypsin in aqueous extracts. Test experiments indicated that on extraction of 5 g of powder with 50 ml of water at 0°, the activity against HPLA[2] amounted approximately to 5% of the potential activity and the total procarboxypeptidase (after activation with trypsin) to approximately 2.8 mg/ml.

DEAE-cellulose was a product of Brown and Company, stated by the manufacturer to have a capacity of 0.9 meq/mg. The adsorbent was washed once at 5° with 0.25 M NaCl-0.50 M sodium hydroxide, then at room temperature with several changes of distilled water before being equilibrated with the starting buffer.

Diisopropylphosphorofluoridate (DFP) was prepared by Dr. Elias Awad in this laboratory. The inhibitor was generally used undiluted and its concentration was assumed to be 5 M.

Hippuryl-DL-phenyllactic acid (HPLA) was prepared by Mr. William O. McClure by a modification of the procedure of Eiduson *et al.* (1950).

All salts were reagent grade and distilled water was used throughout the preparations.

Trypsin was a twice-crystallized preparation containing 50% MgSO₄, obtained from the Worthington Biochemical Corporation (lot 509).

Carboxypeptidase A was a five-times-recrystallized preparation isolated by Dr. J.-P. Bargetzi according to the procedure of Allan *et al.* (1963).

Nagarse was obtained from Biddle-Sawyer Co., New York.

1-Fluoro-2,4-dinitrobenzene (FDNB) was a preparation from Matheson, Coleman, and Bell (lot 370219), and was used without further purification.

Assay Procedures.—Procarboxypeptidase A was first activated with trypsin. Where precise values were desired for the determination of specific activity, the

zymogen was activated at 37° with trypsin-procarboxypeptidase mole ratios of 1:3 or 1:5, at pH 6.5, and pH 8.0, respectively. Maximum activity was approached slowly, particularly with the purified zymogen, and it was necessary to continue the activation for 6–8 hours. Where less precision was required, as in locating the activity in the column effluent, activation was continued for 1–2 hours at a standard trypsin concentration of 100 μg/ml. After activation of the zymogen, carboxypeptidase activity was measured against the ester substrate HPLA, and in some cases against the peptide CGP. For assay with HPLA, the substrate concentration was 0.01 M in 0.045 M NaCl–0.005 M sodium barbital buffer, pH 7.5. Since only the L isomer is hydrolyzed, the true substrate concentration was 0.005 M. Hydrolysis was followed at 25° by continuous titration with 0.1 M sodium hydroxide at pH 7.5, 25°, using a Neilands-Cannon (1955) pH stat.

The assay for endopeptidase activity was performed using the synthetic substrate ATEE. The substrate solution had the following composition: 0.010 M ATEE, 0.001 M Tris HCl, pH 8.0, 0.1 M KCl. The reaction was followed at 25° by continuous titration at pH 8.0 with 0.1 N NaOH in a Neilands-Cannon pH stat. Initial velocities were used for calculation of activity.

For the assays with HPLA or ATEE, the activity is expressed as the uptake of OH^- in μeq per minute per mg of protein (protein concentration determined from $E^{0.1\%}_{280\,m\mu} = 1.9$ [Keller *et al.*, 1956]).

Since the hydrolysis of HPLA by crystalline carboxypeptidase A causes an uptake of 200 μeq of OH^- per minute per mg of protein under the same assay conditions given above, it is possible to calculate the rate constant to be expected in the procarboxypeptidase assay if one molecule of active carboxypeptidase A is produced from one molecule of procarboxypeptidase A-S6. If the molecular weights of procarboxypeptidase A-S6 and carboxypeptidase are taken as 87,000 and 34,300, respectively, then calculation gives

$$k^0 = 200 \times (34,300/87,000) =$$
$$79.1 \text{ μeq } OH^-/\text{min/mg protein}$$

for procarboxypeptidase A-S6. This factor was used to determine the procarboxypeptidase content (expressed as mg/ml) of effluent chromatographic fractions. However, the maximal activity observed upon activation of purified procarboxypeptidase A-S6 gave a rate constant approximately 80% of that calculated.

Other Methods.—Sedimentation analyses were carried out in the Spinco Model E ultracentrifuge and free boundary electrophoresis in the Spinco Model H apparatus.

Dinitrophenylation was carried out according to the procedure of Levy (1955) at pH 8, 25°. The reaction was followed in a Neilands-Cannon pH stat equipped with the reaction vessel described by Dixon and Wade (1958).

The DNP-proteins were hydrolyzed in constant-boiling HCl for 16 hours at 105° in evacuated, sealed tubes. Ether-soluble DNP-amino acids were separated and identified by two-dimensional paper chromatography using *tert*-amyl alcohol, saturated with 3% aqueous ammonia in the first dimension and 1.5 M sodium phosphate in the second. Multi-Sheet Frame Chromatanks (Shandon Scientific Company, Ltd.) were used. The separation of DNP-aspartic acid and DNP-glutamic acid was carried out as described by Levy (1955). The hydrolyzates of DNP-proteins were examined for water-soluble DNP-amino acids by the procedure of Rovery *et al.* (1953). Literature values (Levy, 1955) of molar extinction coefficients of the DNP-amino acids in 1% sodium bicarbonate were used for the quantitative estimation of DNP-amino acids, after their elution from the paper chromatograms.

The amount of DNP-protein from which the DNP-amino acids so estimated had been derived was measured by amino acid analysis of an aliquot of the water-soluble fraction of the hydrolyzate, using internal standards as described by Walsh and Brown (1962). In calculating the amount of DNP-protein taken for hydrolysis, the values for those amino acids which react with FDNB—lysine, histidine, tyrosine, cysteine, and the N-terminal amino acids—were suitably corrected on the basis of the known amino acid composition of the native protein. Corrected values for serine and threonine, amino acids normally destroyed by acid hydrolysis, were also used. In the amino acid analysis on the short column, ε-DNP-lysine is eluted after arginine.[3] The same ninhydrin color factor was assumed for ε-DNP-lysine as for arginine. The yield of ε-DNP-lysine serves to a first approximation as an internal standard which may be used for the correction of the yields of the amino terminal DNP-amino acids. The yields of ε-DNP-lysine in the analysis of samples "O-2," "N-2," and "N-3" (Table IV) were 0.70, 0.51, and 0.71 of theoretical, respectively.

DNP-proteins were oxidized according to the procedure of Bettelheim (1955). Identification and determination of DNP-cysteic acid in the hydrolyzate of the oxidized DNP-protein was carried out by the following procedure: the hydrolyzate was extracted with ether and then evaporated to dryness *in vacuo*, over sodium hydroxide. The residue was dissolved in a small volume of ethanol and applied to Whatman No. 3 paper. Control standards of DNP-cysteic acid and ε-DNP-lysine were also applied to the paper. The DNP-cysteic acid in the sample was resolved by electrophoresis in 0.1 M pyridine formate buffer, pH 2.1, at 40 v/cm for 30 minutes. The DNP-cysteic acid was then eluted with 1% sodium bicarbonate and its absorbancy at 360 mμ measured against a control paper blank. Regeneration of amino acids from their respective DNP derivatives was carried out with concentrated aqueous ammonia at 105° in evacuated, sealed tubes, and the regenerated amino acids were identified using the high voltage paper electrophoresis-paper chromatographic technique of Richmond and Hartley (1959).

Carboxyl terminal groups were determined by measuring the release of amino acids after incubation of procarboxypeptidase A with carboxypeptidase A. To remove free amino acids from the substrate, a concentrated solution of procarboxypeptidase was dialyzed in thin cellophane tubings overnight against a suspension of mixed-bed resin (Amberlite 400 and Dowex 50) with stirring. The dialyzed solution was lyophilized and dissolved in 0.2 M sodium barbital buffer, pH 7.5, containing 0.1 M NaCl to which 1 M DFP in isopropanol was added to a final concentration of 10^{-3} M. Crystals of carboxypeptidase were washed three times with a large volume of water, and then dissolved in a

[3] The ε-DNP-lysine peak is asymmetric and therefore cannot be integrated by the usual triangular approximation (Spackman *et al.*, 1958). The integration method based on the sum of the heights of dots throughout the fraction (Spackman *et al.*, 1958) is satisfactory but laborious because of the width of the fractions. A convenient method for integration of very small or asymmetric peaks is to trace out the profile of the fraction on paper, cut out the tracing, and weigh the paper. The relation of weight to area is determined by weighing the paper of known area. This procedure is quite satisfactory for peaks having absorbancy less than 0.1.

small volume of 1 M NaCl. After dilution with the pH 7.5 buffer just described, the enzyme and zymogen mixtures were allowed to incubate for time intervals ranging from 0 to 6 hours. After precipitation of the proteins with trichloroacetic acid (15% final concentration) the soluble amino acids were estimated by reaction with the ninhydrin reagent (Matheson et al., 1961) and the individual amino acids were identified either by paper electrophoresis-paper chromatograph (Richmond and Hartley, 1959), or with the Spinco Amino Acid Analyzer.

Amino acid analysis was performed in a Spinco Amino Acid Analyzer, Model 120, according to Spackman et al. (1958). Proteins were hydrolyzed with constant-boiling HCl in evacuated, sealed tubes for varying time periods at 104°. Half-cystine was determined as cysteic acid in hydrolyzates of protein oxidized by performic acid at 0° according to Hirs (1956). An internal standard containing a mixture of β-thienylalanine and α-amino-β-guanidinopropionic acid was added to correct for the variability between short and long column determination as described by Walsh and Brown (1962).

RESULTS

The steps involved in a typical experimental procedure, using 100 g of acetone powder as starting material, are described below.

Extraction.—100 g of pancreatic acetone powder was suspended in 1 liter of cold distilled water containing 10^{-3} M DFP. Two or three drops of n-octanol were added to reduce foaming, and the suspension was stirred at 4° for 4 hours. The suspension was centrifuged for 45 minutes at 18,000 rpm in the No. 21 rotor of the Spinco Model L ultracentrifuge, and the residue was discarded. No further treatment of the extract was necessary before chromatography.

Chromatography.—DEAE-cellulose was equilibrated with 0.005 M potassium phosphate, diluted from 1 M stock, pH 8.0. A 3.4 × 60-cm column was packed under 5 psi and washed with 1 liter or more of 0.005 M potassium phosphate buffer, pH 8.0. The extract, 800–850 ml, was pumped onto the column at 100 ml/hour. The column was washed at 100 ml/hour with 1 liter of 0.05 M potassium phosphate buffer, pH 8.0, containing 10^{-3} M DFP. A linear gradient was then applied from 0.06 M to 0.40 M potassium phosphate, pH 8.0; 2.2 liters of each buffer was used, and 20-ml fractions were collected. The column was monitored by optical density measurements at 280 mμ or by biuret color and by activity against HPLA after tryptic activation (Fig. 1).

It may be observed from the accompanying elution diagram (Fig. 1) that direct chromatography of the crude aqueous extract of pancreas acetone powders resolves two components which hydrolyze HPLA after tryptic activation, but not before. The first component, which emerges after the breakthrough fraction, is procarboxypeptidase B, which was described by Folk (1956) and has been subsequently purified and characterized by Pechère et al. (1958), Wintersberger et al. (1962), and Cox et al. (1962). The second peak is in fact a mixture of two distinct forms of procarboxypeptidase A which can be distinguished by their sedimentation behavior as well as by their chromatographic properties (Brown et al., 1963b).

A broad peak containing potential carboxypeptidase activity emerged at a buffer concentration of about 0.10–0.15 M. Fractions were pooled which covered the peak from just before its maximum to its trailing edge, as shown in Figure 1. The leading third of the procarboxypeptidase peak, which is discarded, contains
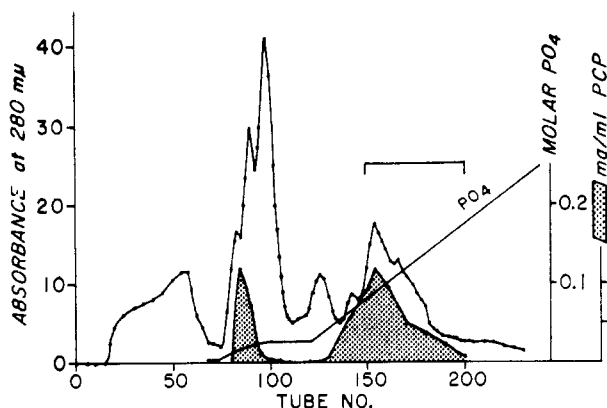

FIG. 1.—Chromatography on DEAE-cellulose of an aqueous extract of acetone powder of beef pancreas glands, in potassium phosphate buffer, pH 8.0. Experimental conditions are described in the text. Shaded area: activity against HPLA after tryptic activation. The bracket denotes the fractions that were pooled for subsequent rechromatography.

procarboxypeptidase A-S5 (Brown et al., 1963b), and a great deal of inactive protein.

Ammonium Sulfate Precipitation.—The fractions containing procarboxypeptidase A were pooled and 0.283 g of solid ammonium sulfate was added per ml of eluate (40% saturation in water at 0°). The precipitate was collected by centrifugation for 30 minutes at 8,000 rpm in the No. 858 rotor of the International centrifuge HR-1, and was then dissolved in about 100 ml of water. The brownish-yellow solution was brought to 10^{-3} M DFP and stirred at 4° for 1–2 hours. The solution was then dialyzed for 1–2 days against repeated changes of 0.005 M potassium phosphate buffer, pH 8.0. Any material remaining undissolved was removed by centrifugation.

Rechromatography.—The dialyzed procarboxypeptidase solution was applied to a 3 × 20-cm DEAE-cellulose column previously equilibrated with 0.005 M potassium phosphate, pH 8.0. The procarboxypeptidase was then eluted with a linear gradient from 0.005 to 0.33 M potassium phosphate, pH 8.0 (Fig. 2). A substantial proportion of the colored impurities remained irreversibly absorbed near the top of the column and did not appear in the elution profile. Fractions containing procarboxypeptidase were pooled as shown by the bracket in Figure 2, and brought

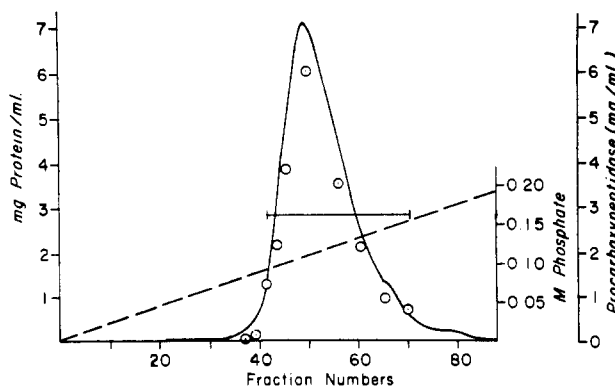FIG. 2.—Rechromatography on DEAE-cellulose of procarboxypeptidase in potassium phosphate, pH 8.0. Experimental conditions are described in the text. Open circles: activity against HPLA after tryptic activation, converted to procarboxypeptidase concentration. Solid line: protein concentration, measured by optical density at 280 mμ, using $E_{280}^{0.1\%} = 1.9$. Dashed line: calculated phosphate gradient.

to 10^{-3} M DFP. After stirring in the cold for 1–2 hours, the solution was dialyzed for 2 days against repeated changes of water. The dialyzed solution was lyophilized and the protein was stored at −20°; 1400–1700 mg of protein was recovered, accounting for 60–70% of the activity against HPLA detectable after tryptic activation of the crude extract.

Amino Acid Composition.—The amino acid composition of a typical preparation of procarboxypeptidase A-S6 is given in Table I. In view of the importance of the amino acid composition, particularly as it relates to the composition of fractions isolated from disaggregation mixtures (Brown et al., 1963b), amino acid analysis was extended to a number of preparations under as nearly identical conditions as possible. The results given in Table II show nearly identical composition for all preparations. The variations in serine, threonine, valine, and isoleucine are not significant, since the yields depend on the time of hydrolysis, nor are those in half-cystine significant since, when determined as such, the recovery of this amino acid is not reproducible.

The most probable values for the amino acid composition of procarboxypeptidase A-S6 are given in Table III. The data are based on the mean compositions given in Tables I and II. Threonine and serine are corrected by extrapolation to zero time of hydrolysis and the values for valine and isoleucine are those reached after 120 hours of hydrolysis. Half-cystine is based on determination of cysteic acid in hydrolyzates of performic acid–oxidized preparations of "MY 7" and "MY 24." Tryptophan was estimated from the tryosine-tryptophan ratio of 1:01 as determined spectrophotometrically (Brown et al., 1963a).

Amino Terminal Groups.—The best preparations of procarboxypeptidase A isolated by the present procedure yield stoichiometrically significant amounts of DNP-aspartic acid and di-DNP-lysine. Performic-acid oxidized samples yield, in addition, DNP-cysteic acid. The results of these analyses are presented in Table IV. No other DNP-amino acid could be found in stoichiometrically significant quantities in preparations isolated from acetone powders that meet

TABLE I
AMINO ACID ANALYSIS OF PROCARBOXYPEPTIDASE A-S6[a]

Preparation	16	36	80	156	Mean or Corrected
	┌──Hours of Hydrolysis──┐				
Lysine	28.9	27.9	27.6	28.8	28.3
Histidine	18.7	18.3	18.3	18.5	18.4
Arginine	25.6	26.5	27.0	26.7	26.5
Aspartic acid	78.1	77.8	77.0	79.4	78.3
Threonine	49.1	47.1	44.6	38.6	49.9
Serine	60.7	56.6	43.9	27.6	64.6
Glutamic acid	79.1	77.3	78.6	80.4	78.9
Proline	40.1	36.2	37.3	39.9	38.3
Glycine	68.2	67.8	68.2	67.6	68.1
Alanine	54.7	55.2	54.2	55.4	54.9
Half-cystine	15.5	13.8	13.6	7.1	16.6
Valine	48.2	55.7	59.9	61.2	60.4
Methionine	5.3	6.0	5.5	6.5	5.7
Isoleucine	37.3	41.5	45.0	45.3	45.0
Leucine	61.2	58.8	63.6	63.9	61.8
Tyrosine	28.3	28.4	28.9	27.6	28.3
Phenylalanine	27.0	29.1	30.0	30.6	29.1

[a] Preparation "JRB 20." The data are expressed as residues per molecule, assuming a molecular weight of 87,000. The mean or corrected composition represents the mean of the data with appropriate corrections for time of hydrolysis.

TABLE II
COMPARISON OF AMINO ACID ANALYSES OF VARIOUS PREPARATIONS OF PROCARBOXYPEPTIDASE A-S6[a]

Preparation	"MY 7"	"MY 24"	"JRB 20"	"DJC"
	┌────Hours of Hydrolysis────┐			
	120	144	156	120
Lysine	30.4	31.6	28.8	30.0
Histidine	19.5	19.3	18.5	19.2
Arginine	28.9	27.5	26.7	29.1
Aspartic acid	77.1	82.2	79.4	76.9
Threonine	44.1	40.8	38.6	43.7
Serine	41.7	34.1	27.6	41.4
Glutamic acid	79.4	83.2	80.4	78.2
Proline	41.8	43.7	38.3	40.9
Glycine	66.3	68.0	67.6	64.6
Alanine	53.5	53.5	55.4	53.5
Half-cystine	6.6	12.6	7.1	5.5
Valine	57.9	56.3	61.2	57.9
Methionine	6.3	5.8	6.5	6.9
Isoleucine	45.7	47.7	45.3	43.4
Leucine	66.1	68.5	63.9	65.5
Tyrosine	29.8	29.6	27.6	30.2
Phenylalanine	29.6	32.0	30.6	29.1

[a] The data are expressed as residues per molecule assuming a molecular weight of 87,000.

the criteria specified in the experimental section of this report (preparations O-2, N-2, and N-3, in Table IV). However, when DFP was omitted during isolation (preparations "DC-1," "MY-24"), additional end groups were found, i.e., DNP-valine and DNP-threonine, respectively (Table IV).

Carboxyl Terminal Groups.—The results of digestion with procarboxypeptidase A-S6 with carboxypeptidase are shown in Figure 3. In decreasing order of appearance, the main products are leucine, tyrosine, valine, and phenylalanine. After 8 hours of digestion, the mixture was dinitrophenylated and examined for free DNP-amino acids and for DNP end groups of the protein. The results indicated that no new amino terminal groups had been formed and that the free amino acids were solely due to the action of carboxypeptidase. The allocation of carboxyl terminal groups among the polypeptide chains of procarboxypeptidase A-S6, of course, is not evident from the present data and must await further investigation.

Sedimentation Coefficient, Partial Specific Volume, and Molecular Weight.—In accordance with previous measurements by Keller et al. (1956), the present

TABLE III
CORRECTED AMINO ACID COMPOSITION OF PROCARBOXYPEPTIDASE A-S6[a]

Amino Acid		Amino Acid	
Lysine	29.7	Alanine	53.4
Histidine	19.4	Cysteic acid[c]	18.3
Arginine	28.6	Valine[b]	57.9
Aspartic acid	76.6	Methionine	6.5
Threonine[b]	49.2	Isoleucine[b]	44.6
Serine[b]	61.7	Leucine	65.1
Glutamic acid	77.8	Tyrosine	29.4
Proline	39.1	Phenylalanine	29.4
Glycine	66.0	Tryptophan[d]	29.1

[a] The data are expressed as residues per molecule, assuming a molecular weight of 87,000. The composition represents the mean based on amino acid analyses of five different preparations (see Tables I and II). [b] Threonine, serine, valine, and isoleucine were corrected for the time of hydrolysis. [c] Half-cystine was determined separately on two different preparations as cysteic acid. [d] Tryptophan was estimated from the tyrosine-tryptophan ratio of 1:01 as determined spectrophotometrically from the acid-base absorbancies at 294 mμ.

TABLE IV

AMINO TERMINAL RESIDUES IN PREPARATIONS OF PROCARBOXYPEPTIDASE A-S6[a]

| Prepara-tion | DNP-Amino Acids | | | | |
	Lysine	Aspartic Acid	Half-Cystine	Threo-nine	Valine
O-2*	0.70	0.50	+	–	–
N-2*	0.71	0.42	+	–	–
N-3*	0.83	0.52	+	–	–
DC-1	0.56	0.58	+	–	0.44
MY-24	0.53	0.49	+	0.44	–

[a] The data are expressed as N-terminal residues per molecule of procarboxypeptidase A, assuming a molecular weight of 87,000. The data in rows marked with an asterisk are corrected on the basis of the amount of DNP-lysine recovered in the aqueous phase (see under Methods). All other data are uncorrected. + denotes present, – absent. The amount of half-cystine was separately determined on another preparation, after performic acid oxidation of the DNP-protein and was found to be 0.7 residue per molecule.

preparation of procarboxypeptidase A was found to give a symmetrical boundary in the ultracentrifuge (Fig. 4). The sedimentation coefficient decreases with increasing protein concentration (Fig. 5), and linear extrapolation to infinite dilution gives a value of 6.12 Svedberg units.

The apparent specific volume of the protein had not been previously determined. In order to obtain a reliable value, the densities of the solutions of protein were determined in a parallel-stem pycnometer of the type described by Fox (1955), nominal volume 10 ml. The temperature was set at 20°, nominal value, and variation was read from a Beckman differential thermometer. Two determinations at each protein concentration were obtained by readjusting the level in the calibrated stems for each measurement. The mean value of the apparent density was used to calculate the density corrected for weighing in air. Concentrations were determined either directly by absorbancy measurements at 280 mμ, or by taking the concentration of the most concentrated stock solution as that obtained from absorbancy measurements and calculating the concentrations of the diluted samples
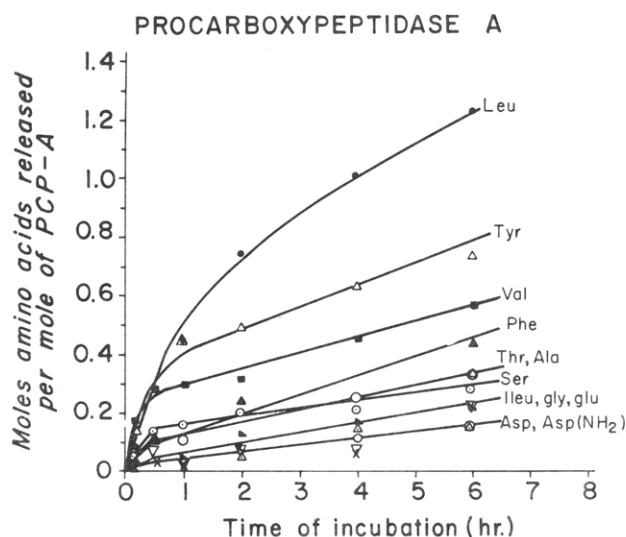
PROCARBOXYPEPTIDASE A

FIG. 3.—Effect of carboxypeptidase A on procarboxypeptidase A-S6. Substrate concentration 0.138 μmoles/ml. Substrate-enzyme mole ratio 12:1. Solvent, 0.02 M sodium Veronal–0.1 M NaCl, pH 7.5, 25°.
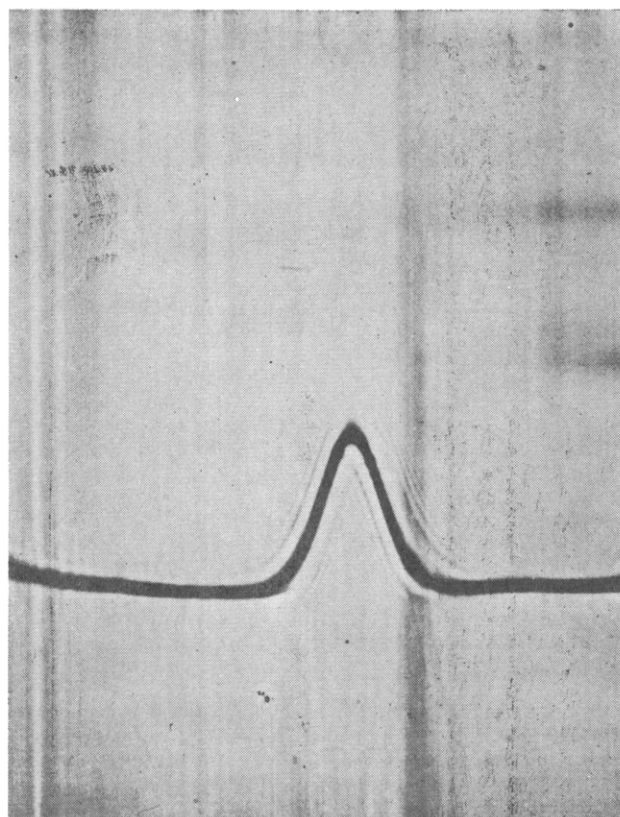

FIG. 4.—Sedimentation pattern of procarboxypeptidase A-S6 in 0.01 M KCl–0.01 M potassium phosphate buffer, pH 6.6, after 72 minutes of sedimentation at 39,780 rpm. Protein concentration 1.0%, bar angle 70°.
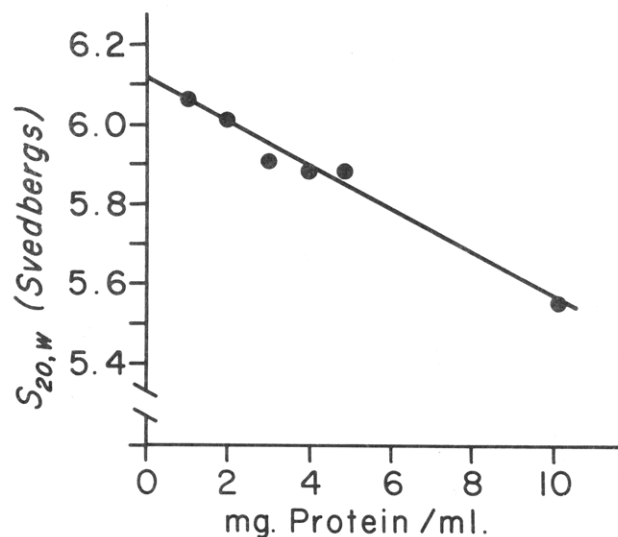

FIG. 5.—Concentration dependence of sedimentation coefficient of procarboxypeptidase A-S6 in 0.01 M HCl–0.01 M potassium phosphate buffer, pH 6.6, normalized to the viscosity and density of water at 20°.

from values of ρ for the solvent and the stock solution by means of weighing. Measurements were made at four concentrations and a mean value of $\bar{v} = 0.720 \pm 0.016$ was calculated as given by Schachman (1957).

The molecular weight was calculated with the equation of Svedberg and Pedersen (1940) using $s_{20,w} = 6.12$ S, $D_{20,w} = 6.23 \times 10^{-7}$ cm^2 sec^{-1} (Keller *et al.*, 1958), and $\bar{v} = 0.72$, yielding a molecular weight of 87,080.
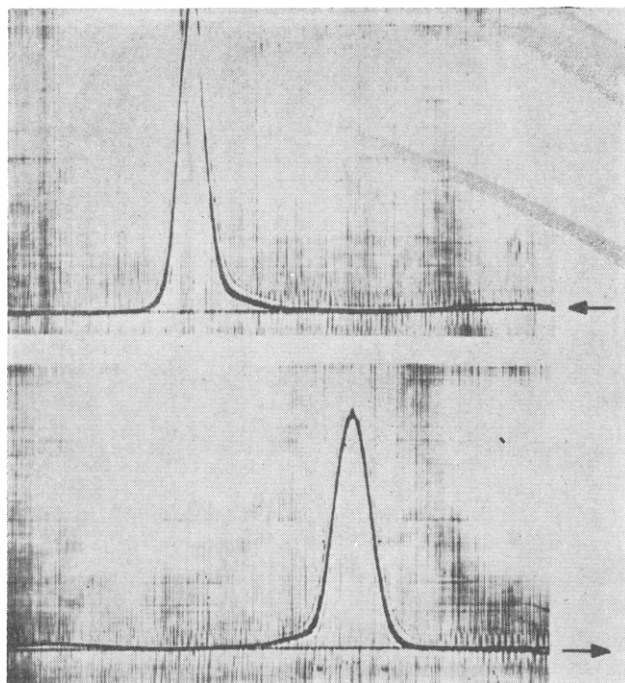
FIG. 6.—Moving boundary electrophoresis of procarboxy-peptidase A-S6, in 0.1 M sodium cacodylate–0.15 M NaCl, pH 6.7. Field strength, 2.90 v cm^{-1}. Direction of migration as indicated by the arrows. Mobilities: ascending, 4.1, and descending, 4.0×10^{-5} cm^2 volt^{-1} sec^{-1}.

FIG. 7.—Chromatography of procarboxypeptidase A-S6 on DEAE-cellulose in potassium phosphate buffers of pH 8.0 and pH 6.5, respectively.

FIG. 8.—Activation of procarboxypeptidase A-S6 by trypsin at 37°: left, in 0.04 M phosphate buffer, pH 8.0; right, in 0.04 M phosphate buffer, pH 6.5. For further details, see the text.

Molecular weights were also determined by short-column sedimentation equilibrium (Van Holde and Baldwin, 1958) and calculated by the procedure of Deranleau (1961). The concentration of the protein after dialysis against a 0.04 M potassium phosphate buffer, pH 6.75, was 17.61 mg/ml. For each experiment 50 μl of solution and 100 μl of FC 43 fluorocarbon (Schachman, 1957) were placed in one limb of a double-sector cell and 160 μl of solvent was placed in the other limb. Experiments were done at three concentrations, i.e., 17.61, 11.14, and 5.87 mg/ml. The calculated molecular weight was 84,100 $\pm$ 2,290 at a 95% confidence level with a standard deviation of 3,400.

Homogeneity.—Procarboxypeptidase A-S6 purified by the present procedure is homogeneous in free boundary electrophoresis and in chromatography. The electrophoretic pattern obtained in 0.1 M sodium cacodylate buffer containing 0.15 M NaCl at pH 6.7 is shown in Figure 6, and indicates a single nearly symmetrical boundary. Chromatography of the purified zymogen on DEAE-cellulose at pH 6.5 and pH 8.0 also indicates a high degree of purity (Fig. 7).

Activation.—As previously observed by Keller *et al.* (1956, 1958), conversion of procarboxypeptidase to carboxypeptidase proceeds slowly even at a trypsin-procarboxypeptidase mole ratio of 1:5. Typical activation curves at pH 8.0 and pH 6.5 are shown in Figure 8. The conditions of activation at pH 8.0 are comparable to those of Keller *et al.* (1958), i.e., 0.74 mg/ml of procarboxypeptidase A-S6 and 0.045 mg/ml of trypsin. At pH 6.5, where activation also occurs, the zymogen concentration was 12 mg/ml and the trypsin concentration was 2 mg/ml. The effect of trypsin concentration on the rate of the activation is shown in Figure 9 for procarboxypeptidase-trypsin mole ratios of approximately 140:1, 2.5:1, 1.25:1, and 0.63:1, respectively. Within this range, the rate of activation increases with increasing trypsin concentration and, except for the lowest trypsin concentration, nearly the
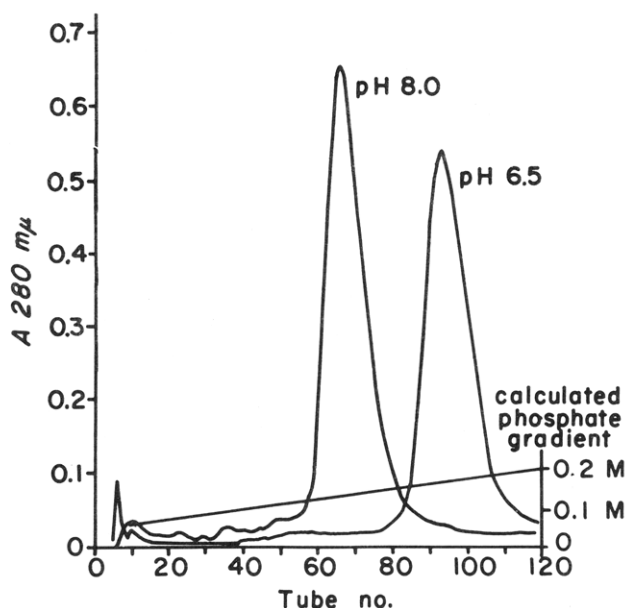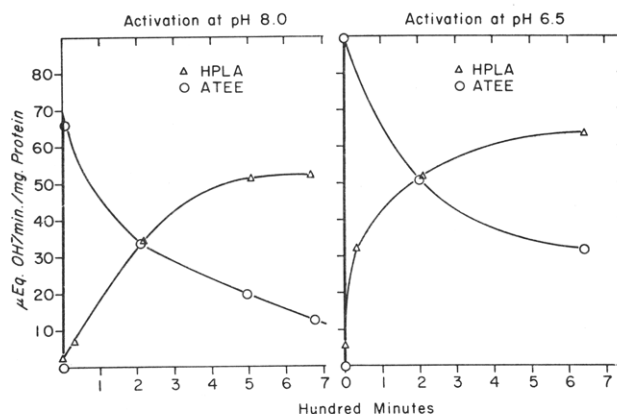
same carboxypeptidase A activity is reached after 700 minutes of incubation. Figure 9 also includes points for endopeptidase activity (ATEE as substrate) after 2 and 700 minutes of activation. Essentially the same results were obtained in Tris-HCl buffer as in phosphate buffers. Activation could also be achieved using Nagarse instead of trypsin as catalyst, but after the same maximal activity was reached further incubation with Nagarse caused a decline in carboxypeptidase A activity.

As discussed under assay procedures, the activity toward HPLA to be expected for the formation of one molecule of carboxypeptidase A from one molecule of procarboxypeptidase A-S6, is 79.1 μeq/min/mg protein. The maximal activities observed for a large number of preparations under various conditions of activation were 75–80% of the expected value. The reasons for this discrepancy are not clear and may require more detailed determinations of certain of the molecular parameters of the zymogen, including molecular weight, extinction coefficient, and metal analyses. Precise determinations of the maximal
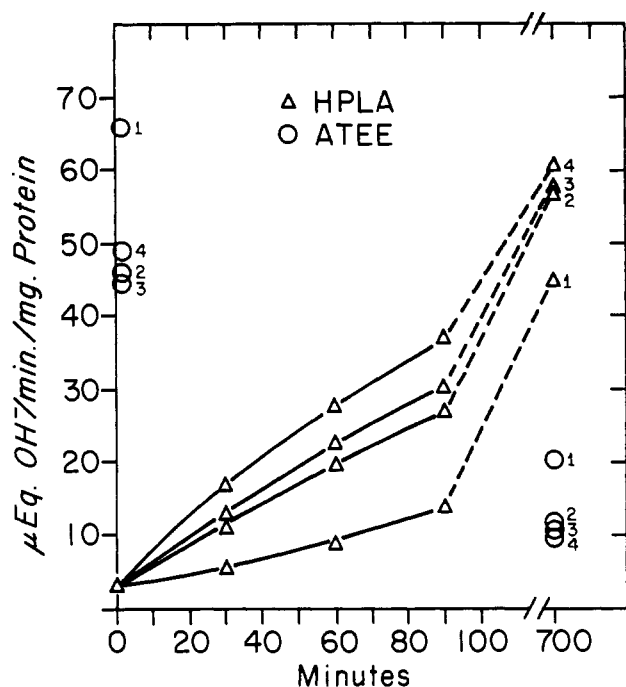
FIG. 9.—Effect of trypsin concentration on the activation of procarboxypeptidase A-S6 in a 0.04 M phosphate buffer, pH 7.8 at 37°. Concentration of procarboxypeptidase 860 μg/ml. Trypsin concentration in experiments corresponding to curves 1 to 4 was 3.2, 92, 184, and 368 μg/ml, respectively. △ activity against HPLA; O activity against ATEE. Activities against ATEE assays were made only after 2 and 700 minutes of activation. For further details, see the text.

endopeptidase activity, under the conditions described by Keller et al. (1958) also remain to be made, and to be correlated with titrations of the "active centers," with cinnamoylimidazole (Schonbaum et al., 1961), and with DFP[32].

Procarboxypeptidase A has some carboxypeptidase A activity even before tryptic activation. In the best preparations, the activity of the zymogen against HPLA is about 5–6% of that obtainable after complete tryptic activation. The same level of activity has been observed in the aqueous extracts of acetone powder before fractionation and may well be an intrinsic property of the proenzyme itself.

DISCUSSION

Procarboxypeptidase A-S6 isolated as described in the present paper has the same molecular and enzymatic properties as that previously described by Keller et al. (1956, 1958). The present isolation procedure is considerably simpler than those previously used, and gives much better and more consistent yields. The procedure described in the experimental section of this report may be varied in a number of ways. The first chromatography of the crude extract may be carried out using Tris–sodium chloride buffers instead of phosphate. The standard procedure in that case is to use 0.01 M Tris-HCl at pH 8 in concentrations of NaCl increasing from 0 to 0.4 M. Alternatively, the procedure of Wintersberger et al. (1962) may be followed. The aqueous extract of the acetone powder is dialyzed before chromatography and the chromatography is carried out in 0.01 M Tris-HCl buffer at pH 7. After elution of procarboxypepti-

dase B by 0.02 M NaCl in a pH 7 Tris buffer, procarboxypeptidase A may be eluted by a gradient of NaCl from 0 to 0.4 M in the same buffer. Chromatography of the crude extract by either of these methods seems to be as good a procedure as the standard one described herein. In the rechromatography, however, phosphate buffers cannot be replaced by Tris whether the first chromatography was done in phosphate or in Tris. If the first chromatography is carried out in phosphate buffers at pH 6.5 instead of pH 8.0, a slower sedimenting impurity is frequently observed, which is difficult to remove even by subsequent chromatography at pH 8.0. However, this impurity can be removed by isoelectric precipitation at pH 5.2 (Keller et al., 1956), the impurity remaining in the supernatant solution. An alternative preparative procedure which has been used with success involves the first chromatography on DEAE-cellulose at pH 8.0, and the second chromatography at pH 6.5.

The most critical aspect of the present procedure is the quality of the acetone powders. It is necessary that these powders contain essentially no active trypsin and that the intrinsic carboxypeptidase activity of the aqueous extract not exceed 5% of the total carboxypeptidase activity obtainable after activation. The production of such entirely "unactivated" powders requires some care in dealing with the tissue. The glands must be collected and quickly chilled in ice; after trimming, the glands should be frozen in solid carbon dioxide and kept frozen until the acetone drying is done. Once suitable powders have been prepared, they are quite stable during storage at −20°. While different lots of powder may give rather different elution profiles, the position of the procarboxypeptidase peak in the column effluent is fairly constant. It is frequently possible to prepare procarboxypeptidase from powders of lower quality. The material so isolated is generally identical to the best preparations in sedimentation and chromatography behavior, and in specific activity after tryptic activation. Invariably, however, such preparations seem to contain breaks in the peptide chains as evidenced by the appearance of amino terminal residues in addition to those of lysine, aspartic acid, and cystine. It is not unusual to detect significant amounts of only one or two additional amino terminal groups, notably threonine or valine, rather than a spectrum of all the possible products. Even preparations of very carefully handled acetone powders may contain such additional end groups unless the preparations have been exposed to DFP at several stages during the isolation procedure. Thus the restriction of free α-amino groups to those of lysine, aspartic acid (or asparagine), and cystine (or cysteine) seems the most sensitive criterion of a good preparation.

The high degree of homogeneity in electrophoresis, sedimentation, and chromatography of the present preparation is fully in accord with the properties of the zymogen as prepared by Keller et al. (1958). The present data, in addition, provide a more complete characterization of the protein in terms of amino acid composition and a revision of the molecular weight. The latter results from a new experimental value of the partial specific volume and from a revision of the sedimentation coefficient. The value of approximately 87,400 obtained from these data agrees well with the value obtained in the present work from sedimentation equilibrium measurements. The presence of three amino terminal residues is in accord with the view that procarboxypeptidase A-S6 is composed of three polypeptide chains and that the molecule is a complex of three different protein subunits (Brown et al., 1961, 1963a).

REFERENCES

Allan, B. J., Keller, P. J., Neurath, H. (1963), in preparation.

Bargetzi, J.-P., unpublished experiments.

Bettelheim, F. R. (1955), *J. Biol. Chem. 212*, 235.

Brown, J. R., Cox, D. J., Greenshields, R. N., Walsh, K. A., Yamaski, M., and Neurath, H. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1554.

Brown, J. R., Greenshields, R. N., Yamasaki, M., and Neurath, H. (1963a), *Biochemistry 2*, 867 (this issue).

Brown, J. R., Yamasaki, M., and Neurath, H. (1963b), *Biochemistry 2*, 877 (this issue).

Cox, D. J., Wintersberger, E., and Neurath, H. (1962), *Biochemistry 1*, 1082.

Deranleau, D. A. (1961), *Biochim. Biophys. Acta 54*, 345.

Dixon, G. H., and Wade, R. D. (1958), *Science 127*, 338.

Eiduson, S., Camien, M. N., and Dunn, M. S. (1950), *Arch. Biochem. 29*, 302.

Fischer, E. H., and Stein, E. A. (1954), *Arch. Sci. (Geneva) 7*, 131.

Folk, J. E. (1956), *J. Am. Chem. Soc. 78*, 3541.

Fox, J. B., Jr. (1955), Ph.D. dissertation, University of Washington.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Keller, P. J., Cohen, E., and Neurath, H. (1956), *J. Biol. Chem. 223*, 457.

Keller, P. J., Cohen, E., and Neurath, H. (1958), *J. Biol. Chem. 230*, 905.

Levy, A. L. (1955), in Methods of Biochemical Analysis, Vol. II, Glick, D., ed., New York, Interscience, p. 359.

Levy, A. L., Geschwind, I. I., and Li, C. H. (1955), *J. Biol. Chem. 213*, 187.

Matheson, A. T., Tigane, E., and Hanes, C. S. (1961), *Can. J. Biochem. Physiol. 39*, 417.

Neilands, J. B., and Cannon, M. D. (1955), *Anal. Chem. 27*, 29.

Pechère, J.-F., Dixon, G. H., Maybury, R. H., and Neurath, H. (1958), *J. Biol. Chem. 233*, 1364.

Richmond, V., and Hartley, B. S. (1959), *Nature 184*, 1869.

Rovery, M., Fabre, C., and Desnuelle, P. (1953), *Biochim. Biophys. Acta 12*, 547.

Schachman, H. K. (1957), in Methods in Enzymology, Vol. IV, Colowick, S. P., and Kaplan, N. O., eds., New York, Academic.

Schonbaum, G. R., Zerner, B., and Bender, M. L. (1961), *J. Biol. Chem. 236*, 2930.

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

Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, New York, Oxford University Press.

Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.

Walsh, K. A., and Brown, J. R. (1962), *Biochim. Biophys. Acta 58*, 596.

Wintersberger, E., Cox, D. J., and Neurath, H. (1962), *Biochemistry 1*, 1069.