A New Form of Bovine Pancreatic Procarboxypeptidase A*

JAMES R. BROWN,† MAKOTO YAMASAKI,‡ AND HANS NEURATH

From the Department of Biochemistry, University of Washington, Seattle, Washington

Received April 19, 1963

Bovine pancreatic procarboxypeptidase A-S5 has been isolated from aqueous extracts of acetone powder of pancreas glands. Similar to procarboxypeptidase A, previously described (Keller *et al.*, 1956, 1958a; Yamasaki *et al.*, 1963), the present zymogen, upon activation with trypsin, gives rise to carboxypeptidase A, an endopeptidase which hydrolyzes the substrate acetyl-L-tyrosine ethyl ester. The new form of procarboxypeptidase A has a sedimentation coefficient of approximately 5 S in contrast to the zymogen previously described which has a sedimentation coefficient of 6 S and is composed of two (I and II) rather than three subunits. Disaggregation into fractions I and II has been accomplished in alkaline solutions and the fractions have been separated by chromatographic procedures and characterized chemically and enzymatically. Evidence is presented here that these fractions are identical to the corresponding fractions of the larger form of procarboxypeptidase A (Brown *et al.*, 1961, 1963). A general scheme is presented for the interrelation of the various subunits to procarboxypeptidases A.

Recent work on the chemical structure and biological functions of bovine pancreatic procarboxypeptidase A has shown that this protein is the precursor of two enzymes, i.e., carboxypeptidase A and an endopeptidase which hydrolyzes certain substrates for chymotrypsin, such as acetyl-L-tyrosine ethyl ester (Keller *et al.*, 1956, 1958a). These two enzymatic activities are associated with different subunits[1] which constitute the zymogen: carboxypeptidase A with subunit I and the endopeptidase with subunit II. The two subunits and a third one, seemingly devoid of biological function, are firmly associated in native procarboxypeptidase, but disaggregation can be brought about in concentrated urea solutions or in aqueous solutions above *p*H 10 (Brown *et al.*, 1961, 1963). Intact procarboxypeptidase, having a sedimentation coefficient of 6 S and a molecular weight of approximately 87,000 (Yamasaki *et al.*, 1963) has been found in bovine pancreatic juice, as well as in aqueous extracts of acetone powders prepared from bovine pancreas glands (Keller *et al.*, 1956, 1958a,b).

When fractions obtained by chromatography of aqueous extracts of pancreatic acetone powders on DEAE-cellulose were tested for potential procarboxypeptidase activity, a new form of the zymogen was discovered having a sedimentation coefficient of 5 instead of 6 Svedberg units. This new form of the zymogen was found to emerge just ahead of the main fraction, and like the latter, yielded on activation both carboxypeptidase A and endopeptidase activities. Having a lower sedimentation coefficient and molecular weight than the main component and containing two rather than three *N*-terminal groups, it was concluded that subunit III of the procarboxypeptidase previously described was missing in this instance. The isolation, activation, and disaggregation of this new zymogen is the subject of the present report.

MATERIALS AND METHODS

Acetone powders of freshly collected bovine pancreas glands were obtained through the courtesy of the Lilly Research Laboratories, Eli Lilly and Company, or else were prepared by the methods previously described (Fischer and Stein, 1954; Keller *et al.*, 1956).

DEAE-cellulose with a capacity of 0.9 meq per gram of resin was a reagent grade product of the Brown Company (Selectacel-DEAE, lot 1096). In early preparations, type 20, lot 1036 was used. The adsorbent was washed and equilibrated as previously described (Wintersberger *et al.*, 1962).

Diisopropylphosphorofluoridate (DFP)[2] was a product synthesized by Dr. Elias Awad or was purchased from Aldrich Chemical Company, Inc.

Trypsin was a twice-crystallized product containing 50% $MgSO_4$ obtained from Worthington Biochemicals.

Hippuryl-DL-phenyllactic acid (HPLA) was prepared as the sodium salt by Mr. W. O. McClure by a modification of the procedure of Snoke *et al.* (1948).

Acetyl-L-tyrosine ethyl ester (ATEE) was obtained from Mann Fine Chemicals.

All salts used were reagent grade.

The *p*H was measured at room temperature with a glass electrode in conjunction with a Radiometer Type PHM 22p *p*H meter.

Amino Acid Analysis was performed on a Beckman-Spinco Model 120 amino acid analyzer according to Spackman *et al.* (1958). Samples were hydrolyzed with constant boiling HCl, in evacuated sealed Pyrex tubes at 104° for specified times. Destruction of threonine and serine was corrected by extrapolation to zero-time hydrolysis. Maximum recoveries of valine and isoleucine were obtained after 84–192 hours hydrolysis. Half-cystine + cysteine was determined as cysteic acid in hydrolyzates of proteins oxidized with performic acid at 0° as described by Hirs (1956). Internal standards were included in samples for amino acid analysis as described by Walsh and Brown (1962).

Tryptophan content was calculated from the tyrosine content and the tyrosine-tryptophan ratio as estimated spectrophotometrically (Brown *et al.*, 1963).

* Part of a dissertation presented by James R. Brown to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This work was presented in part before the Pacific Slope Biochemical Conference held in Seattle on September 6 and 7, 1962. This work has been supported in part by the Public Health Service (RG-04617), by the American Cancer Society (P-79), and by the Office of Naval Research (NONR-477-04).

† Present address: Laboratory of Molecular Biology, University of Cambridge, Cambridge, England.

‡ Present address: College of General Education, University of Tokyo, Department of Chemistry, Tokyo, Japan.

[1] For a definition of the terms "subunit" and "fraction," see the accompanying paper (Brown *et al.*, 1963).

[2] The following abbreviations are used: DFP = diisopropylphosphorofluoridate; HPLA = hippuryl-DL-phenyllactic acid; ATEE = acetyl-L-tyrosine ethyl ester; FDNB = 1-fluoro-2,4-dinitrobenzene; DNP = dinitrophenyl.

Dinitrophenylation of the proteins was carried out in aqueous medium at *p*H 8 according to the method of Levy *et al.* (1955), as detailed in a recent publication from this laboratory (Cox *et al.*, 1962).

Enzymatic Assays.—Endopeptidase activity was measured against the ester substrate ATEE. Carboxypeptidase activity was measured against the ester HPLA. The details of these assay procedures are given in an accompanying report (Yamasaki *et al.*, 1963).

Enzymatic activities were expressed as μeq OH^- per minute per mg protein (protein concentration determined from $E^{0.1\%}_{280\,m\mu} = 1.9$).

Moving boundary electrophoresis was performed in a Spinco Model H instrument.

Sedimentation analysis was performed in a Spinco Model E ultracentrifuge. Sedimentation coefficients were calculated from least square slopes as discussed by Schachman (1957). The observed coefficients were corrected to 20° and water as the solvent using equation (6) of Schachman. The viscosities and densities of the various buffers were calculated from the density and viscosity increments for the separate components given by Svedberg and Pedersen (1940).

Results

Isolation of Procarboxypeptidase

Aqueous extracts of acetone powder were prepared by suspending one part of powder (usually 100 g) in 10 parts (g/g) of distilled water to which DFP had been added to a final concentration of 1×10^{-3} M. After 4–12 hours' extraction in the cold room, the extract was centrifuged for 20 minutes at 20,000 rpm in a No. 21 rotor of a Spinco Model L ultracentrifuge, and the clear supernatant pumped onto a column (3.4 cm × 60 cm) of DEAE-cellulose previously equilibrated with a buffer containing 0.01 M potassium phosphate buffer, *p*H 6.5.

After emergence of the "breakthrough" peak, a linear phosphate gradient was applied, as shown in Figure 1. The fractions emerging in tubes No. 150–200 were pooled and rechromatographed at *p*H 6.5, as shown in Figure 2. The fraction corresponding to

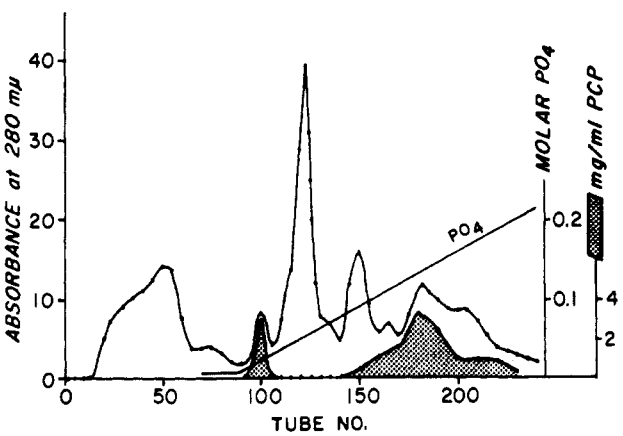

FIG. 1.—Chromatography on DEAE-cellulose of aqueous extracts of acetone powder of bovine pancreas glands. Experimental conditions were as follows: 820 ml of extract from 100 g acetone powder applied to a column 3.4 cm × 60 cm. Tube volume 25 ml, flow rate 100 ml/hour. Initial buffer, 0.01 M potassium phosphate, *p*H 6.5, linear phosphate gradient as shown. The shaded areas represent procarboxypeptidase as determined by activity against HPLA after activation with trypsin. The fraction giving a peak at tube 100 is procarboxypeptidase B (Wintersberger *et al.*, 1962). The main peak (tubes 150 to 200) represents procarboxypeptidase A-S5 and -S6.

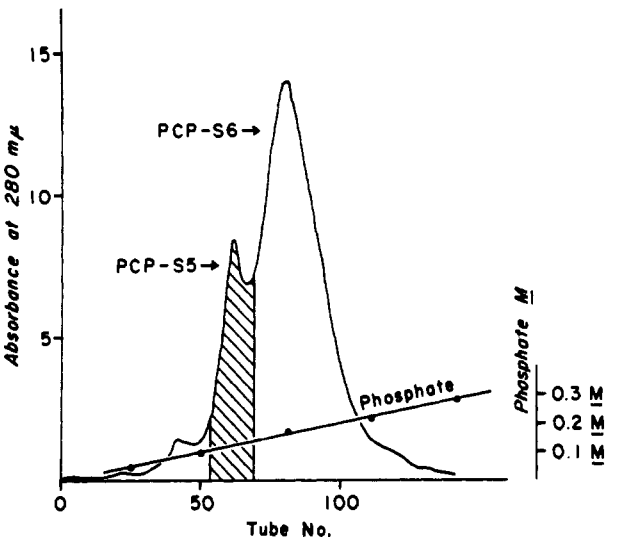

FIG. 2.—Chromatography on DEAE-cellulose at *p*H 6.5 of fractions corresponding to tubes 150 to 200 of the first chromatography (see Fig. 1). The experimental conditions were similar to those given in the legend of Figure 1. Partial resolution of procarboxypeptidase A-S5 and -S6 is evident. The fractions corresponding to the hatched area were pooled and again subjected to chromatography as shown in Figure 3.

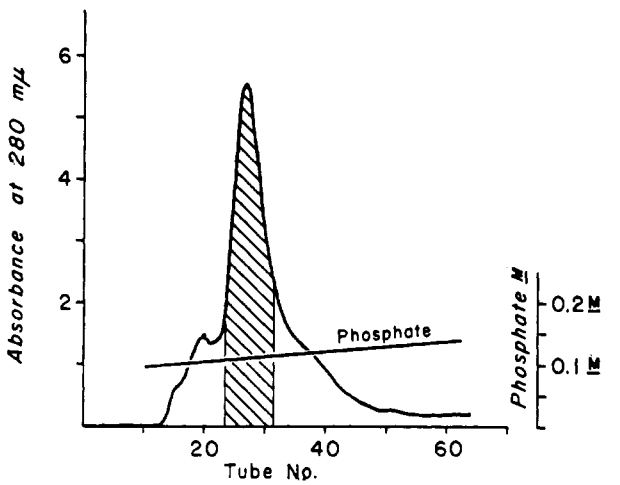

FIG. 3.—Third chromatography of procarboxypeptidase A-S5 on DEAE-cellulose, at *p*H 8.0. Column dimensions 2 cm × 37 cm, tube volume 8 ml. Fractions corresponding to the hatched area were pooled and subjected to one additional chromatography as shown in Figure 4.

the hatched area was found to have predominantly a sedimentation coefficient of 5 S and after pooling was subjected again to chromatography, this time at *p*H 8.0, under the conditions shown in Figure 3. A final rechromatography of the protein corresponding to the hatched area in Figure 3 gave a preparation which was chromatographically homogeneous as shown in Figure 4. Most of the studies reported in this paper were carried out with material prepared in this fashion, which will be referred to as procarboxypeptidase A-S5. In a typical experiment, 100 g of acetone powder yielded 300 mg of chromatographically pure procarboxypeptidase A-S5.

Keller *et al.* (1956) have described a procedure for the isolation of procarboxypeptidase from acetone powders based on ammonium sulfate fractionation and isoelectric precipitation. Some of the experiments reported in this paper were carried out with material

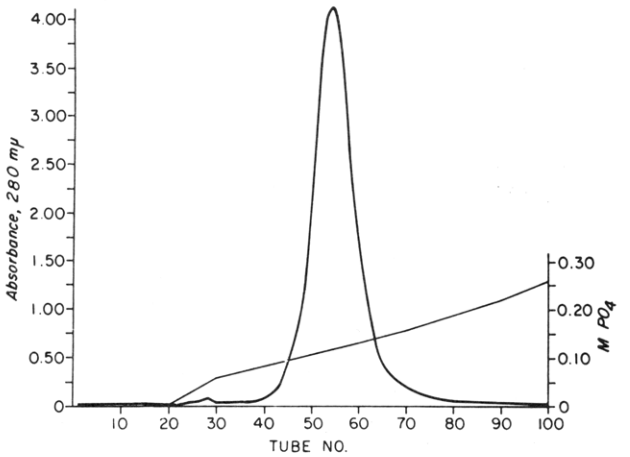

FIG. 4.—Fourth chromatography of procarboxypeptidase A-S5 on DEAE-cellulose at *p*H 6.5. Column dimensions 2 cm × 30 cm, tube volume 8 ml.

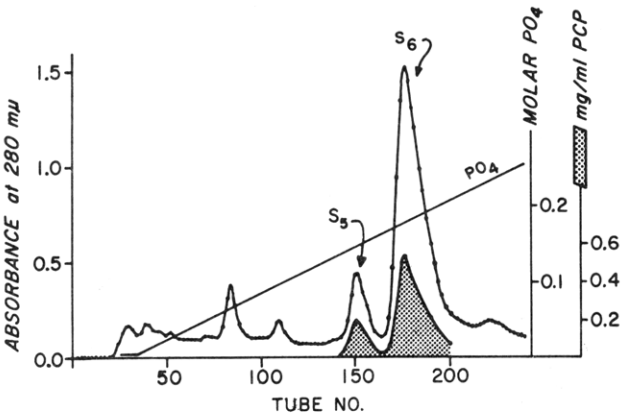

FIG. 5.—Chromatography on DEAE-cellulose at *p*H 8.0 of partially purified procarboxypeptidase A obtained by ammonium sulfate fractionation and isoelectric precipitation as described by Keller *et al.* (1956). Experimental conditions were as follows: 470 mg protein; column dimensions 3.5 × 32 cm, tube volume 15 ml. Initial buffer, 0.005 M potassium phosphate, *p*H 8.0; linear phosphate gradient. The cross-hatched areas indicate procarboxypeptidase A as determined by activity against HPLA after activation with tryspin. The two forms of procarboxypeptidase A, S5 and S6, are clearly resolved, in a ratio (optical density) of approximately 1:4.

prepared by a similar procedure prior to chromatography on DEAE-cellulose. The last step of isoelectric precipitation and extraction was repeated twice, yielding a fraction which was approximately 50% pure as judged by enzymatic assay. When this material was subjected to chromatography on an analytical scale, an elution pattern shown in Figure 5 was obtained. Large-scale chromatography and rechromatography at *p*H 8, followed by chromatography at *p*H 6.5 under the conditions just described, yielded a material which behaved like the material obtained by direct chromatography of the aqueous extract of the acetone powder. To denote the difference in procedures employed, the former material will be referred to as PCP'-S5.

Properties of Procarboxypeptidase A-S5

Sedimentation Analysis and Molecular Weight.—Preparations of procarboxypeptidase A-S5 obtained by either procedure appear homogeneous in the ultracentrifuge, as shown in Figure 6. The dependence of the sedimentation coefficient on the concentration is characteristic of a nonaggregating system as shown in

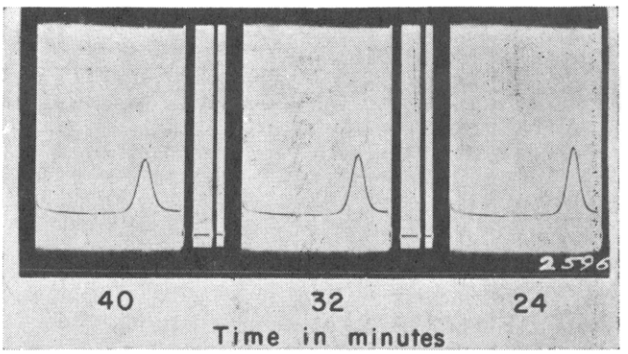

FIG. 6.—Sedimenting boundary of procarboxypeptidase A-S5. Protein concentration 11 mg/ml, solvent 0.04 M potassium phosphate buffer, *p*H 6.5. Conditions: 59,503 rpm; bar angle 70°, temperature 20°. s_{20w} = 4.68 S.

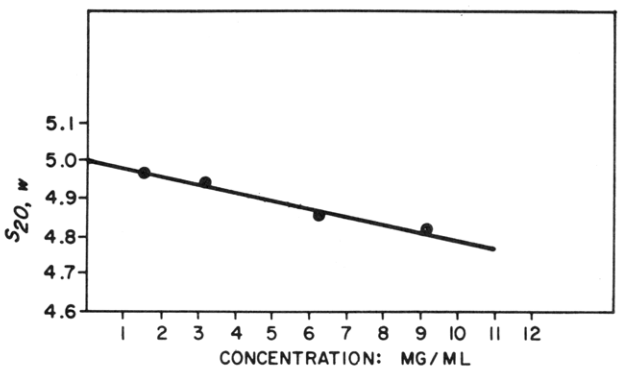

FIG. 7.—Concentration dependence of the sedimentation coefficient of procarboxypeptidase A-S5. Experiments in 0.04 M potassium phosphate buffer, *p*H 6.5, containing 0.0005 M DFP, 59,780 rpm, 19°.

Figure 7. Extrapolation to zero protein concentration gives a sedimentation coefficient of $s_{20^0,w}$ = 5.0 S.

Measurements of the sedimentation equilibrium, by the method of Yphantis (1960) in 0.04 M potassium phosphate buffer *p*H 6.5, containing 0.0005 M DFP, gave values of 71,400, 73,600, and 72,600, with a mean of 72,500. This value for the molecular weight is higher than that calculated from the sum of fraction I (36,000) and fraction II (27,000), i.e., 63,000. The molecular weight calculated on the basis of 6.00 methionine residues per molecule is 67,500. Precise determinations of molecular weight now in progress in this laboratory may resolve these differences.

Electrophoresis.—Moving boundary electrophoresis of procarboxypeptidase A-S5 gave patterns characteristic of a homogeneous protein. In a 0.1 M Tris-HCl buffer *p*H 7.5 at 0°, the mobility of the ascending boundary was -4.4×10^{-5} cm^2 volt^{-1} sec^{-1}.

Amino Acid Anslysis.—The amino acid composition of procarboxypeptidase A-S5 prepared by direct chromatography was analyzed in detail. Two preparations were used, #43 and #44, and analyses were also made on an oxidized sample of one of these (#44). The data are presented in Table I as amino acid residues per molecule, assuming a molecular weight of 64,000. The corrected composition is given in Table II.

Analogous analyses were performed on a preparation of PCP'-S5. The results in general were similar and hence are not presented here.

N-Terminal Analysis.—Reaction of procarboxypeptidase A-S5 with FDNB was carried out at *p*H 8.0 in 0.1 M KCl according to the method of Levy *et al.* (1955). At the end of the reaction, the DNP-protein was

TABLE I

AMINO ACID ANALYSIS OF PROCARBOXYPEPTIDASE A-S5[a]

	Prep. 44		Hours Hydrolysis			Prep. 43			Oxidized[b] Prep. 44
Amino Acid	17.5	24	40	84	192	24	40	84	19
Lysine	23.1	25.2	24.4	23.9	25.1	24.1	24.9	25.1	24.1
Histidine	12.7	13.8	13.6	13.2	14.0	14.0	14.1	14.5	13.3
Arginine	22.8	24.1	23.7	23.0	24.1	24.6	24.4	23.9	23.2
Aspartic acid	54.4	53.7	54.2	54.2	53.8	54.3	52.9	54.0	56.7
Threonine	37.4	38.0	36.8	35.4	32.8	38.4	37.3	35.6	37.8
Serine	39.7	39.5	38.5	33.4	28.0	38.9	38.6	34.6	40.2
Glutamic acid	60.3	60.2	60.4	59.7	60.7	59.6	61.2	60.2	60.6
Proline	24.5	24.1	23.8	25.1	25.1	24.4	25.3	25.3	23.3
Glycine	44.1	44.2	44.2	44.1	44.1	44.0	44.1	44.0	44.2
Alanine	38.3	39.0	38.1	39.1	38.4	39.1	38.8	38.8	39.0
Half-cystine	5.27	4.13	6.25	6.43	2.97	4.71	5.21	4.79	9.66
Valine	33.7	36.3	37.6	40.2	40.1	34.8	38.5	39.6	34.9
Methionine	5.84	5.85	5.42	5.57	5.56	5.83	5.81	5.64	5.09
Isoleucine	26.7	29.6	30.2	31.0	33.5	30.4	30.5	31.8	28.4
Leucine	46.8	48.3	47.8	47.3	46.8	49.2	48.4	47.4	47.8
Tyrosine	23.9	23.9	24.1	23.4	23.4	23.7	24.1	24.0	—
Phenylalanine	24.3	24.7	25.0	24.7	24.7	24.5	25.2	24.7	24.5

[a] The data are given as amino acid residues per molecule assuming a molecular weight of 64,000. [b] In the sample oxidized with performic acid, methionine and half-cystine were determined as methionine sulfone and cysteic acid, respectively.

TABLE II

CORRECTED AMINO ACID COMPOSITION OF PROCARBOXYPEPTIDASE A-S5[a]

Amino Acid	Residues per Molecule	Amino Acid	Residues per Molecule
Lysine	24.2	Alanine	38.8
Histidine	13.7	Half-cystine	9.66
Arginine	23.8	Valine	40.1
Aspartic acid	53.9	Methionine	5.7
Threonine	38.8	Isoleucine	33.5
Serine	43.4	Leucine	47.4
Glutamic acid	60.4	Tyrosine	23.8
Proline	24.5	Phenylalanine	20.6
Glycine	44.1	Tryptophan	19.1

[a] The data are expressed as amino acid residues per molecule of procarboxypeptidase A-S5, assuming a molecular weight of 64,000. Threonine and serine have been corrected for destruction during hydrolysis by extrapolation of composition versus time of hydrolysis to time zero. Valine and isoleucine were based on the analysis of the 192-hour hydrolyzate (Table I). Half-cystine was determined as cysteic acid in the hydrolyzate of procarboxypeptidase A-S5, which was oxidized with performic acid prior to hydrolysis. Tryptophan was estimated from the ratio of tyrosine-tryptophan as determined spectrophotometrically (Brown, 1963). All other amino acids represent mean values calculated from the data in Table I.

TABLE III

AMINO TERMINAL ANALYSES OF PROCARBOXYPEPTIDASE A-S5[a]

Preparation	43	40	44	44 (oxidized)
di-DNP-lysine	0.25	0.44	0.38	0.31
DNP-aspartic + DNP-glutamic acids	0.02	0.11	0.11	Not measured
di-DNP-cystine	Trace	Trace	Trace	Absent
DNP-cysteic acid	Trace	Trace	Trace	0.47

[a] The data are expressed as residues per molecule assuming a molecular weight of 64,000. The quantity of protein was determined by amino acid analysis of the acid aqueous phase of the hydrolyzate of the DNP-protein. No corrections were applied to the data. Determination of ϵ-DNP-lysine in preparation 44 in the Spinco amino acid analyzer (Brown *et al.*, 1963) gave 69% theoretical yield, thus indicating a loss of approximately 30% due to incomplete reaction with FDNB, and destruction during hydrolysis. Preparation 43 was dinitrophenylated in the presence of 0.001 M DFP. Preparation 40 is PCP'-S5. Preparation 44 (oxidized) was dinitrophenylated and then oxidized with performic acid.

precipitated with hydrochloric acid, the precipitate washed with water, acetone, and ether, and dried. Hydrolysis of the DNP-protein with constant-boiling HCl was carried out for 12–24 hours, and the ether-soluble DNP-amino acids subjected to two-dimensional chromatography on Whatman #1 paper using *tert*-amyl alcohol saturated with 3% ammonia in one direction, and 1.5 M sodium phosphate *p*H 6 in the other. After elution with 1% $NaHCO_3$, the absorbance of DNP-amino acids was determined at 360 mμ in a Beckman DK-1 recording spectrophotometer. For purposes of quantitative analysis, the molar extinction of mono-DNP-amino acids was assumed to be 18,000, that of di-DNP-lysine as 30,000 (Levy, 1955). The oxidation of the DNP-protein with performic acid was carried out by the method of Bettelheim (1955). DNP-cysteic acid was determined by high-voltage electrophoresis for 30 minutes in 0.1 M pyridine-formate buffer, *p*H 2.1, at 40 v/cm, in an apparatus described by Michl (1953).

The results of *N*-terminal analysis of procarboxypeptidase A-S5 and PCP'-S5 are given in Table III. The data are calculated for a molecular weight of 64,000 and are expressed without any corrections for losses due to incomplete reaction or destruction during hydrolysis. The data clearly suggest that lysine and half-cystine are the *N*-terminal groups of procarboxypeptidase A-S5 since no other DNP-amino acid has been obtained from this protein in stoichiometrically significant quantities. It is also apparent from the data that there are no significant differences in this regard between the two preparations, procarboxypeptidase A-S5 and PCP'-S5.

Activation.—The activation of procarboxypeptidase A-S5 was followed in two steps: first at 0° trypsin was added in a weight ratio of 1:100 in order to follow the rate of appearance of endopeptidase; in the

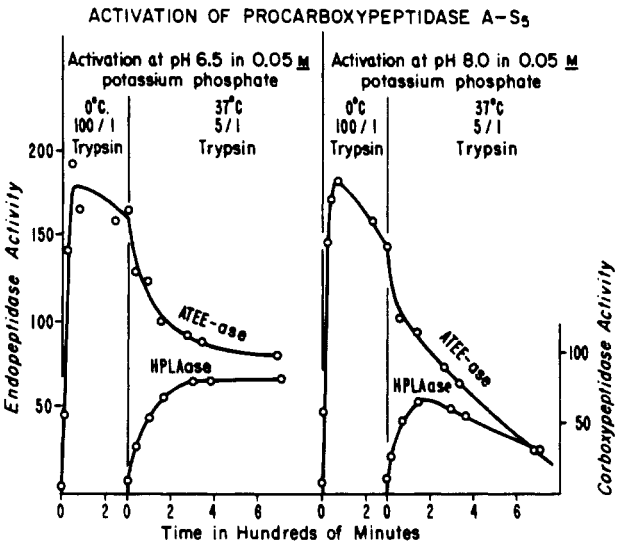

FIG. 8.—Activation of procarboxypeptidase A-S5 at *p*H 6.5 (left), and *p*H 8.0 (right). For details of the conditions of activation, see the text. Units of endopeptidase activity, μeq OH^- per min per mg protein.

second step at 37° the trypsin concentration was increased to a weight ratio of 1:5 on order to measure the rate of appearance of carboxypeptidase-A activity. The experiments were carried out at *p*H 8 and also at *p*H 6.5. The results are presented in Figure 8. It will be noticed that under both conditions, at *p*H 6.5 and at *p*H 8, the formation of the endopeptidase occurs rapidly, reaching a maximum after approximately 30 minutes of incubation. However in the second phase both activities reach a stable level at *p*H 6.5 which is maintained over a period of 5 hours, whereas at *p*H 8.0, under otherwise identical conditions, a steady decline of both activities is noted after the exopeptidase had attained its maximum level.

Keller *et al.* (1956) had found that in the activation of procarboxypeptidase A (S6) at *p*H 8, the sedimentation coefficient changed from 6 S to 3 S and the formation of the 3 S fraction showed a direct correlation with the formation of carboxypeptidase activity. These findings suggested that proteolytic degradation might be an obligatory step in the formation of carboxypeptidase. In the present investigation, the effect of activation on the sedimentation behavior of procarboxypeptidase A-S5 was determined by preparing activation mixtures containing 1% procarboxypeptidase and 0.1% trypsin, to aid the visualization of the sedimenting boundary. The results, shown in Figure 9, indicate that at *p*H 6.5 the sedimentation coefficient changed little during activation, the initial value being 4.6 S, in comparison to 4.8 S after 2 hours and 4.3 S after 8 hours of activation. The reason for the higher value after 2 hours in unknown. The results suggest that at *p*H 6.5 procarboxypeptidase A-S5 can be completely activated without undergoing disaggregation. However, an apparent decrease in the area under the sedimenting boundary was noted, suggesting that some proteolytic degradation into peptides may have occurred under these conditions.

When sedimentation analysis was similarly carried out during activation at *p*H 8.0, the mean sedimentation coefficient decreased only slightly, from 5.0 to 4.5 S, suggesting that under these conditions, too, the S5 protein can be activated without a significant change in molecular mass, whereas procarboxypeptidase A-S6 undergoes fragmentation.

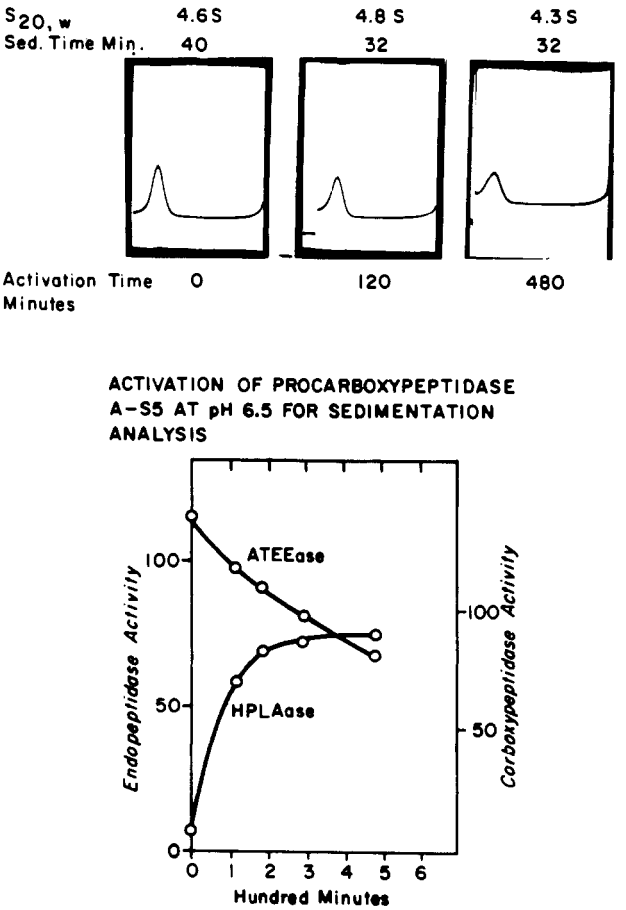

FIG. 9.—Upper: Sedimentation analysis of activation mixtures of procarboxypeptidase A-S5. Activation was carried out at 37° in a 0.04 M potassium phosphate buffer, *p*H 6.5, containing 1% procarboxypeptidase and 0.1% trypsin. The rates of activation observed under these conditions are shown in the lower half

Disaggregation of Procarboxypeptidase A-S5

When solutions of procarboxypeptidase A-S5 in 0.28 M LiCl, 0.1 M glycine buffer at *p*H 10.5, containing 0.01 M DFP were allowed to stand at 20°, disaggregation occurred as evidenced by a time-dependent transformation of a species having initially a sedimentation coefficient of $s_{20}' = 4.4$ S to a species with $s_{20}' = 2.15$ S, the transformation being complete after 48 hours. This is analogous to previous findings for procarboxypeptidase A-S6 (Brown *et al.*, 1963). When the solution was then equilibrated against a *p*H 8 buffer, partial precipitation of the protein occurred. After 30 minutes centrifugation at 17,000 rpm in a high speed head of the International Centrifuge PR-2, a pellet appeared, presumably representing fraction I which in previous work had been shown to be insoluble under these conditions (Brown *et al.*, 1961, 1963).

The supernatant solution was dialyzed against 0.004 M potassium phosphate buffer *p*H 8, and then subjected to chromatography on DEAE-cellulose, with the results shown in Figure 10. It will be noted that the elution pattern presents a single peak. If this elution pattern is compared to that previously published for the disaggregation mixtures for procarboxypeptidase A-S6 (Brown *et al.*, 1961, 1963), it becomes evident that one of the subfractions, namely,

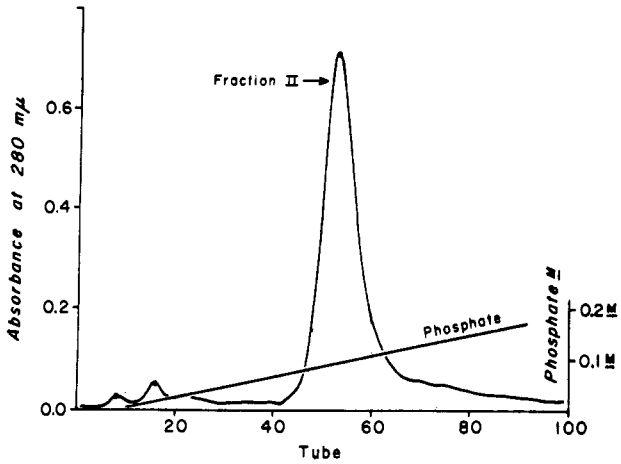

FIG. 10.—Chromatography on DEAE-cellulose of the products of alkaline disaggregation of procarboxypeptidase A-S5. Experimental conditions were as follows: 19 mg protein; column dimensions 0.9 cm × 27 cm; tube volume 4.6 ml; linear gradient of potassium phosphate buffer, *p*H 8.0.

fraction III, is altogether absent from the present system.

Properties of Subunits

The alkaline disaggregation experiment just described indicates that procarboxypeptidase A-S5 is a complex of two protein subunits; however, a more detailed examination of the products of disaggregation is required in order to identify and correlate them with those previously described.

Fraction I.—This fraction, as the corresponding fraction obtained from procarboxypeptidase A-S6, becomes denatured and precipitated under conditions of disaggregation and hence cannot be tested for potential enzymatic activity. However, chemical evidence to be presented below clearly indicates its correspondence to fraction I prepared from procarboxypeptidase A-S6.

N-Terminal Analysis.—As previously indicated, procarboxypeptidase A-S5 contains only lysine and half-cystine as *N*-terminal groups. Since previous work on procarboxypeptidase A-S6 indicates that the *N*-terminal group of fraction I is aspartic acid (or asparagine) and that of fraction II half-cystine, the presence of an *N*-terminal lysine group in the S5 protein was indeed unexpected.

When fraction I was examined by the FDNB method, using the procedure given in the experimental section of this report, only DNP-aspartic acid could be found in the ether phase in stoichiometrically significant quantities. In addition, smaller amounts of DNP-alanine, serine and threonine, and di-DNP-lysine were found, as shown in Table IV. These results are strictly comparable to those previously obtained for the *N*-terminal groups of fraction I isolated from procarboxypeptidase A-S6. Since, in the intact zymogen, procarboxypeptidase A-S5, the end group corresponding to subunit I appears to be lysine, it follows that during alkaline disaggregation, the *N*-terminal lysine, either as such or in the form of a lysyl peptide, is lost, giving way to an aspartic acid or asparagine residue in that position. In view of the importance of the *N*-terminal sequence of carboxypeptidase as it relates both to the method of preparation of this enzyme (Sampath Kumar *et al.*, 1963) and to the binding of zinc (Vallee *et al.*, 1963), a more detailed consideration of the present findings will be deferred for a later occasion.

TABLE IV

AMINO TERMINAL ANALYSIS OF FRACTION I FROM PROCARBOXYPEPTIDASE A-S6 AND -S5[a]

DNP-Amino Acids	Fraction I-S6	Fraction I-S5
Aspartic acid	0.48	0.51
di-Lysine	0.15	0.09
Alanine	0.14	0.12
Serine	0.10	0.17
Threonine	0.09	0.11

[a] The data are expressed as end-group residues per molecule, assuming a molecular weight of 36,000. The quantitation was based on amino acid analysis of the acid soluble phase. No corrections have been applied.

Amino Acid Composition.—After extraction of the ether-soluble DNP derivatives, the acid hydrolyzate of DNP-fraction I was examined in the amino acid analyzer. The results given in Table V indicate that the composition in terms of acidic and neutral amino acids is comparable to that previously given for an acid hydrolyzate of DNP-fraction I prepared from procarboxypeptidase A-S6. It is doubtful that the small differences observed for threonine, serine, glutamic acid, alanine, leucine, and isoleucine, are of significance.

Fraction II.—Fraction II, as obtained by chromatography of the disaggregation mixture of procarboxypeptidase A-S5, appears in the ultracentrifuge as a single symmetrical peak having a sedimentation coefficient $s_{20,w}$ of 2.8 S (protein concentration 0.63%).

N-Terminal Analysis.—When fraction II was dinitrophenylated, oxidized with performic acid, and hydrolyzed, only DNP-cysteic acid was found as amino terminal residue, in a yield of 0.34 residues per molecule, assuming a molecular weight of 27,000 and without any corrections for losses. When ε-DNP-lysine recovered from the hydrolyzate was taken as internal standard, and the calculations based on the known lysine content of fraction II, the yield of DNP-cysteic acid was 0.9 residue per molecule. The ether soluble phase of the acid hydrolyzate of oxidized DNP-fraction II appeared to be devoid of any DNP-amino acids, indicating that half-cystine is the only amino terminal group in this fraction.

Amino Acid Composition.—The amino acid composition of fraction II obtained from procarboxypeptidase A-S5 is presented in Table VI, together with corresponding data obtained from procarboxypeptidase A-S6.

TABLE V

COMPARISON OF AMINO ACID COMPOSITIONS OF DNP-FRACTIONS I FROM PROCARBOXYPEPTIDASE A-S6 AND -S5[a]

Amino Acid	DNP-Fraction I-S6	DNP-Fraction I-S5	Amino Acid	DNP-Fraction I-S6	DNP-Fraction I-S5
Aspartic acid	29.0	29.5	Alanine	20.0	22.0
Threonine	22.9	24.0	Valine	17.2	17.7
Serine	26.3	31.1	Methionine	2.86	2.82
Glutamic acid	28.0	30.0	Isoleucine	22.5	21.2
Proline	12.0	12.0	Leucine	23.5	24.4
Glycine	26.5	26.1	Phenyl-alanine	16.4	16.2

[a] The data are expressed as amino acid residues per molecule, assuming a molecular weight of 36,000. Threonine, serine, valine, and isoleucine have been corrected for time of hydrolysis on the basis of data obtained in the amino acid analysis of procarboxypeptidase A-S5. The samples used for these analyses were from the acid hydrolyzates of the DNP-proteins after the ether-soluble DNP-amino acids were extracted.

TABLE VI

COMPARISON OF AMINO ACID COMPOSITIONS OF FRACTIONS II FROM PROCARBOXYPEPTIDASE A-S6 AND -S5[a]

Amino Acid	Difference	Fraction II-S5[b]	Fraction II-S6
Lysine		7.8	7.5
Histidine		5.2	5.1
Arginine		9.3	8.8
Aspartic acid		25.5	25.5
Threonine		16.8[c]	16.8
Serine		14.4[c]	14.3
Glutamic acid	+3	25.8	22.8
Proline		12.1	11.8
Glycine		22.8	22.8
Alanine	+1	17.1	16.2
Cysteic acid		Not done	8.9
Valine	+1	20.6[b]	19.6
Methionine		1.2	1.0
Isoleucine		13.7[b]	13.5
Leucine	+1	22.0	21.3
Tyrosine		6.7	7.0
Phenylalanine		7.3	7.2
Tryptophan[d]		13.4	14.0

[a] The data are expressed as amino acid residues per molecule assuming a molecular weight of 27,000. [b] The data for fraction II-S5 are from a single analysis with a hydrolysis time of 24 hours. [c] Threonine, serine, valine, and isoleucine of fraction II-S5 have been corrected for hydrolysis time using correction factors found for fraction II-S6. [d] Tryptophan was estimated from the ratio of tyrosine-tryptophan as determined spectrophotometrically (Brown, 1963).

The data for the present preparation have been obtained from a single analysis with a hydrolysis time of 24 hours. Within the limits of the error, the agreement in amino acid composition between the two proteins listed in Table VI is good, lending further support to the assumption that the two proteins of fraction II are identical.

Enzymatic Properties.—Fraction II from procarboxypeptidase A-S5 had no enzymatic activity prior to activation with trypsin. When trypsin was added, endopeptidase but no carboxypeptidase activity appeared. A typical progress curve for this activation is shown in Figure 11.

The specific activity of fraction II obtained from either procarboxypeptidase A-S5 or -S6 was found to be lower than expected from the activities and composition of the parent zymogen. The reason for this apparent loss in activation is not evident at the moment and will be the subject of further studies.

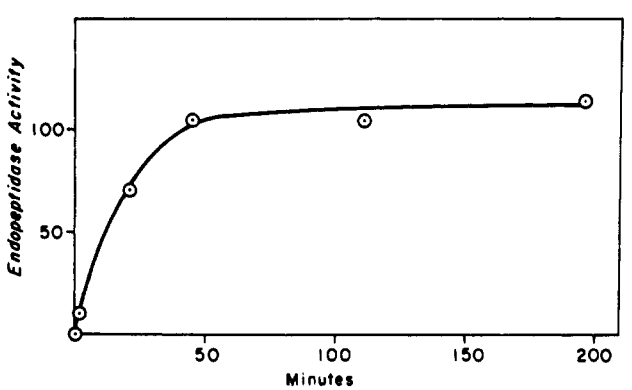

FIG. 11.—Progress of tryptic activation of fraction II. Experimental conditions were as follows: 190 μg per ml fraction II, 0.08 μg per ml tryspin, 0.04 M potassium phosphate buffer, pH 6.5, 25°. For the enzymatic assay, 100 μl of the activation mixture were added to 3.0 ml of a solution of ATEE as described under Methods.

DISCUSSION

The present investigation has provided conclusive evidence that the new form of procarboxypeptidase A is an aggregate of two rather than three subunits. The evidence is based on the sedimentation coefficient and molecular weight of the intact zymogen, and on end-group analysis and amino acid composition of the zymogen and of its disaggregation products. Enzymatic and chemical analysis of the products of disaggregation indicates that the new form of procarboxypeptidase A is composed of subunits I and II of the zymogen previously described (procarboxypeptidase A-S6).

An over-all scheme showing the relationship of the two procarboxypeptidases A, S5 and S6, to the products obtained therefrom by disaggregation and activation is presented in Figure 12, wherein the various proteins are arranged in three columns according to sedimentation coefficient, and in four rows according to enzymatic properties.

Procarboxypeptidase A-S5 is shown in the 5S column, row 1, as a complex of subunits I and II. The various steps of activation of this zymogen are shown as transitions, down the 5S column, leading first to the endopeptidase (row 2) and next to the "dual enzyme" having both endopeptidase and carboxypeptidase-A activities. Since the preparation of carboxypeptidase A from procarboxypeptidase A-S5 has not yet been demonstrated, the transition from the "dual enzyme" to carboxypeptidase A must be considered hypothetical and is so indicated in Figure 12.

The alkaline disaggregation of procarboxypeptidase A-S5 into fractions I and II is shown in row 1 as a transition to the 3S column. Fraction I is represented as a jagged circle to indicate that it has become denatured. The denaturation of subunit I is probably the mechanism which allows the disaggregation to go to completion. The close relationship of fraction I to carboxypeptidase A is indicated by the similarity of the two proteins in their amino acid composition. However, besides being denatured, fraction I also differs from subunit I in amino end group which appears to be lysine for subunit I, but is aspartic acid (or asparagine) for fraction I. Thus, the discovery of conditions which would allow the isolation of undegraded

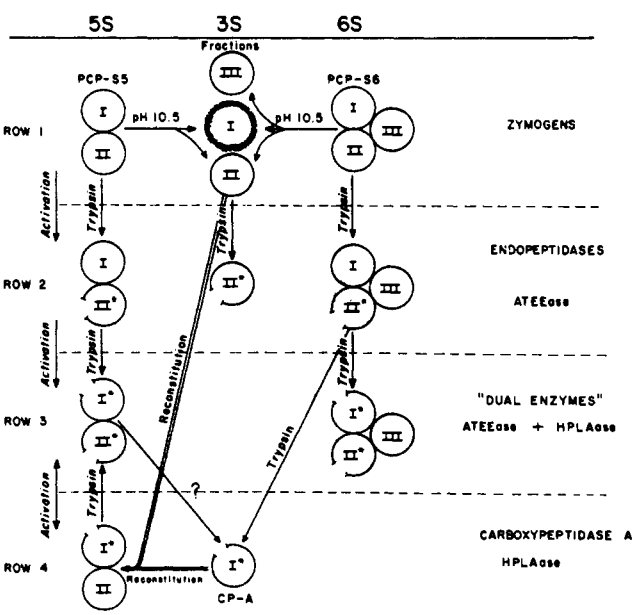

FIG. 12.—Diagrammatic representation of the subunit structure of procarboxypeptidases A-S5 and -S6, including activation, disaggregation, and reconstitution. For details, see the text.

native subunit I, representing the true precursor of carboxypeptidase A, remains one of the important problems for future research. Such studies are now being pursued in this laboratory.

Fraction II appears to be the native zymogen for an endopeptidase which resembles the known chymotrypsins in enzymatic specificity. Thus, fraction II, chymotrypsinogen A (Bettelheim, 1955), and chymotrypsinogen B (Kassell and Laskowski, 1962), all have half-cystine as the amino terminal group. However, fraction II differs significantly in amino acid composition from chymotrypsinogen A or B (Brown *et al.*, 1963). The conversion of fraction II to endopeptidase is shown in Figure 12 as a transition from row 1 to row 2. This step involves the hydrolysis of a peptide bond by trypsin, giving rise to valine as the amino end group of the active enzyme, in contrast to isoleucine in the initial step of activation of chymotrypsinogen A. The observation that activation of procarboxypeptidase A-S5 does not lead to disaggregation suggested that the structural features of subunit I responsible for binding to subunit II are still present in the activated product. It thus seemed possible that a mixture of carboxypeptidase A and of fraction II should recombine spontaneously to form a complex resembling partially activated procarboxypeptidase A-S5. Preliminary experiments indicated this to be, indeed, the case. Thus when fraction II was dissolved in 0.04 M potassium phosphate buffer, *p*H 6.5, and a suspension of crystalline carboxypeptidase A in the same buffer was added, the crystals largely dissolved, and examination of the supernatant solution in the ultracentrifuge indicated the presence of a major and minor boundary with sedimentation coefficients, $s_{20,w}$, of 5.0 S and 2.5 S-respectively. It is worthy of note that both components, carboxypeptidase A and fraction II were derived from procarboxypeptidase A-S6, whereas combination halted at a stage corresponding to an S5 unit, thus giving further support to the view that subunits I and II of procarboxypeptidase A-S5 are identical with the corresponding subunits of the S6 zymogen, and furthermore, that subunits II and III are different proteins. Recombination did not occur when carboxypeptidase A was mixed with chymotrypsinogen B instead of fraction II. The reconstituted protein could be activated with trypsin so as to yield again both carboxypeptidase and endopeptidase activity. This reconstitution of a complex between fraction II and carboxypeptidase A is indicated in Figure 12, by double arrows leading to the product (5 S, row 4), whereas the subsequent activation of the complex with trypsin is shown as a transition from row 4 to row 3, in the 5S column. The reconstitution of a carboxypeptidase with a sedimentation coefficient of 6 S, by mixing the enzyme with fractions II and III, has not yet been investigated.

The alkaline disaggregation of procarboxypeptidase A-S6 into fractions I, II, and III is shown in Figure 12, row 1, as a transition from 6 S to 3 S. Fraction III is presumed to be a zymogen, but attempts to identify an enzymatic activity with this protein, before or after activation by trypsin, have been unsuccessful (Brown *et al.*, 1961). Fraction III was found to have no inhibitory effect on carboxypeptidase A, but the possibility of a complex formation was not investigated. The sedimentation coefficients and amino acid compositions of fractions II and III are similar, thus suggesting that they may be related to one another. However, the fact that disaggregation of procarboxypeptidase A-S5 does not yield any trace of fraction III and the observation that a mixture of carboxypeptidase A and fraction II leads to a complex having a sedimentation coefficient of 5 S rather than 6 S, mitigates against the possibility that subunits II and III are identical (see above) and that fraction III is an artifact of the disaggregation process.

The first step in the activation of procarboxypeptidase A-S6, as of the S5 zymogen, is the formation of an endopeptidase, shown as a transition from row 1 to row 2. It is proposed that all three endopeptidases shown in row 2 are formed by the same mechanism, although the amino terminal group formed during activation has been determined thus far only for fraction II. In earlier studies of the activation of procarboxypeptidase (S6) by Keller *et al.* (1956, 1958a), it was suggested that the conversion of this zymogen to carboxypeptidase A might require the partial degradation of the residual protein (subunits II and III), and no evidence was found for the formation of a protein having both endopeptidase and carboxypeptidase activity. However, when in the present studies activation of procarboxypeptidase A-S6 was carried out at *p*H 6.5, rather than at *p*H 8.0, ultracentrifugal examination of the activation mixtures demonstrated the presence of a major fraction having a sedimentation coefficient of 6 S after 80% of activation had occurred (Fig. 9). This finding indicates the formation of a "dual enzyme" as a major product, analogous to that observed in the activation of procarboxypeptidase A-S5. These results indicate that disaggregation is not an obligatory step for the activation of subunit I in procarboxypeptidase A-S6.

The formation of carboxypeptidase A either from procarboxypeptidase A-S6 or -S5, occurs slowly and requires rather vigorous conditions, i.e., high trypsin-zymogen ratios at a temperature of 37°. In contrast, the activation of procarboxypeptidase A in pancreatic juice occurs more rapidly (Keller *et al.*, 1958b), possibly through the mediation of other pancreatic enzymes. Other zmogen-enzyme transformations occur rapidly under mild conditions (Neurath, 1957), indicating the presence of a particular labile and available peptide bond which is specifically hydrolyzed. More recent studies, of the activation of procarboxypeptidase B (Wintersberger *et al.*, 1962; Cox *et al.*, 1962), indicate that activation of this zymogen also occurs readily and rapidly. On the basis of these observations, one is led to the expectation that the conversion of subunit I to carboxypeptidase A should also be a rapid process. A plausible explanation for the difficulty of conversion of procarboxypeptidase A to carboxypeptidase A is that a specific peptide bond in subunit I which must be hydrolyzed to effect activation is located near the site of binding between subunit I and II, and is thus masked from the action of trypsin until it becomes exposed as the result of nonspecific proteolytic degradation. On the basis of this hypothesis, the monomeric subunit I should be readily converted by trypsin to carboxypeptidase A.

A difficult problem in the present studies is to show that fractions isolated from disaggregation mixtures are identical with the subunits from which they were derived. Some of the problems relating fraction I to subunit I have already been indicated. In the case of fraction II, amino acid composition, amino terminal analysis, enzymatic properties, molecular weight, and ability to associate with carboxypeptidase A all are consistent with the properties to be expected for subunit II. However, the following discrepancies must be considered.

1. The specific activity of fully activated fraction II toward ATEE is only approximately 50% of that expected from the specific activity of fully activated procarboxypeptidase A-S5 or -S6.

2. Reconstitution of a complex between fraction II and carboxypeptidase A was less than expected from the amount of reactants, as if some of fraction II had lost its ability to combine with carboxypeptidase.

3. The methionine content of fraction II is only 35–50% of that expected from the amino acid composition of procarboxypeptidase A-S5 and of fraction I (Tables II and VI).

A resolution of these discrepancies must await further experimentation. However, as a working hypothesis for future research, it may be suggested that the integrity of some of the methionine residues of subunit II is essential for enzymatic activity as well as for the specificity of combination with carboxypeptidase A or with subunit I. This hypothesis receives support from recent observations (Ray *et al.*, 1960; Lawson and Schramm, 1962) that one of the two methionine residues of chymotrypsin A is essential for the activity of this enzyme. A loss of methionine residues during alkaline disaggregation by a process of peptide bond hydrolysis seems unlikely since no traces of new amino terminal groups were found in the isolated fraction II. The loss of a methionyl peptide from the carboxyl terminal portion, however, cannot be ruled out by the present data.

Another possibility is suggested by considering the methionine and half-cystine (the latter determined as cysteic acid) content of fractions I and II, in comparison to procarboxypeptidase A-S5. The pertinent data are summarized below.

	Methionine	Half-Cystine
Procarboxypeptidase A-S5	6	10
Fraction I	3	2
Fraction II	1	10

While the sum of methionine and half-cystine is the same for the zymogen as for fraction I + II, i.e., 16, fractions I + II have an excess of two half-cystine residues and a deficiency of two methionine residues, as compared to procarboxypeptidase A-S5. These results would be consistent with the hypothesis that two of the methionine residues of subunit II were converted during alkaline disaggregation to homocystine or to a related compound such that homocysteic acid is formed during subsequent oxidation by performic acid. Thus two of the residues found in the cysteic acid peak in the amino acid analyzer might have been derived initially from methionine.

It is of interest to consider the significance of two forms of procarboxypeptidase A in the exocrine secretions of the bovine pancreas. Present studies indicate that the two zymogens, procarboxypeptidase A-S6 and procarboxypeptidase A-S5 occur in a ratio of approximately 4:1. While the earlier studies of bovine pancreatic juice by Keller *et al.* (1958b) did not demonstrate the presence of procarboxypeptidase S5, it is of interest to note that a protein fraction (i.e., component 6) having a sedimentation coefficient of approximately 5 S was observed in the chromatographic profile in a position corresponding to procarboxypeptidase A-S5 and in about the same relative quantity. However, for unknown reasons, no enzymatic activity could be associated with this component. It is unlikely that procarboxypeptidase A-S5 is derived from the S6 form by splitting out of subunit III during the purification procedures, since no trace of procarboxypeptidase A-S5 can be seen on rechromatography of purified procarboxypeptidase A-S6. Furthermore, sedimentation analysis and electrophoresis under a variety of conditions show no evidence for the disaggregation of procarboxypeptidase A-S6 and, similarly, no monomeric forms corresponding to either subunits I, II, or III have yet been seen in bovine pancreatic juice or in aqueous extracts of acetone powders of bovine pancreas glands.[3] It is reasonable to assume that subunits I, II, and III are synthesized separately and that the association occurs at a later stage. In view of the preceding considerations of the difficulty of conversion of the aggregated zymogen to carboxypeptidase A, in contrast to the expected rapidity of activation of the subunits, it seems tempting to suggest that a mechanism exists *in vivo* for the rapid disaggregation and activation of the procarboxypeptidases A-S5 and -S6, and for the control of the aggregation-disaggregation reaction. Experimental proof for the operation of such a mechanism has to await further studies which are now in progress.

Acknowledgment

The authors are indebted to Drs. Jean-Pierre Bargetzi, David J. Cox, Patricia J. Keller, and Kenneth A. Walsh for many valuable discussions and advice; to Mr. Roger D. Wade for sedimentation measurements; to Mr. Peter Schneider for amino acid analyses; and to Mr. James A. Freisheim for technical assistance in the preparation of the zymogen.

References

Bettelheim, F. R. (1955), *J. Biol. Chem. 212*, 235.
Brown, J. R. (1963), Ph.D. dissertation, University of Washington.
Brown, J. R., Cox, D. J., Greenshields, R. N., Walsh, K. A., Yamasaki, M., and Neurath, H. (1961), *Proc. Nat. Acad. Sci. U. S., 47*, 1554.
Brown, J. R., Greenshields, R. N., Yamasaki, M., and Neurath, H. (1963), *Biochemistry 2*, 867 (this issue).
Cox, D. J., Wintersberger, E., and Neurath, H. (1962), *Biochemistry 1*, 1082.
Fischer, E. H., and Stein, E. A. (1954), *Arch. Sci. (Geneva), 7*, 31.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Kassell, B., and Laskowski, M. (1962), *J. Biol. Chem. 237*, 413.
Keller, P. J., Cohen, E., and Neurath, H. (1956), *J. Biol. Chem. 223*, 457.
Keller, P. J., Cohen, E., and Neurath, H. (1958a), *J. Biol. Chem. 230*, 905.
Keller, P. J., Cohen, E., and Neurath, H. (1958b), *J. Biol. Chem. 233*, 344.
Lawson, W. B., and Schramm, H. J. (1962), *J. Am. Chem. Soc. 84*, 2017.
Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 359.
Levy, A. L., Geschwind, I. I., and Li, C. H. (1955), *J. Biol. Chem. 213*, 187.
Michl, H. (1953), *Naturwiss. 40*, 390.
Neurath, H. (1957), *Advan. Protein Chem. 12*, 320.
Ray, W. J., Jr., Latham, H. G., Jr., Katsoulis, M., and Koshland, D. R., Jr. (1960), *J. Am. Chem. Soc. 82*, 4743.
Sampath Kumar, K. S. V., Bargetzi, J.-P., Walsh, K. A., and Neurath, H. (1963), in preparation.
Schachman, H. K. (1957), *in* Methods in Enzymology, Vol. IV, Colowick, S. P., and Kaplan, N. O., eds., New York, Academic.
Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, Oxford, Oxford University Press.
Vallee, B. L., Riordan, J. R., and Coleman, J. E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 109.

[3] The possible existence of monomeric forms at high dilutions of pancreatic extracts has not been investigated.

Walsh, K. A., and Brown, J. R. (1962), *Biochim. Biophys. Acta 58*, 596.
Wilcox, P. E., Cohen, E., and Tan, W. (1957), *J. Biol. Chem. 228*, 999.
Wintersberger, E., Cox, D. J., and Neurath, H. (1962), *Biochemistry 1*, 1069.
Yamasaki, M., Brown, J. R., Cox, D. J., Greenshields, R. N., Wade, R. D., and Neurath, H. (1963), *Biochemistry 2*, 859 (this issue).
Yphantis, D. A. (1960), *Ann. N. Y. Acad. Sci. 88*, 586.