The Subunit Structure of Bovine Procarboxypeptidase A-S6.* Chemical Properties and Enzymatic Activities of the Products of Molecular Disaggregation

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Bovine pancreatic procarboxypeptidase A-S6 is composed of three subunits. Molecular disaggregation occurs in urea solutions above 5 M or in aqueous solutions at *p*H 10.5. Fractions produced by alkaline disaggregation have been separated from each other by chromatography on DEAE-cellulose and characterized by amino acid composition, end-group analysis, and, where possible, by potential enzymatic activity. Evidence is presented that fraction I represents a denatured form of the precursor of carboxypeptidase A, and that fraction II is the subunit corresponding to the zymogen form of the endopeptidase activity inherent in procarboxypeptidase A. The biological function of fraction III remains to be elucidated. Among the experimental methods employed in this work, zone electrophoresis on Sephadex gel has been used to fractionate the products of activation by trypsin. The details of this technique are also described in this report.

Bovine pancreatic procarboxypeptidase A-S6 is the zymogen for two distinct enzymes, an endopeptidase which resembles in specificity chymotrypsin, and the exopeptidase carboxypeptidase A (Keller *et al.*, 1956, 1958; Brown *et al.*, 1961). Previous work (Keller *et al.*, 1956, 1958) suggested that the endopeptidase is associated with a molecule having the same molecular weight as the zymogen, whereas the conversion to carboxypeptidase is accompanied by extensive molecular degradation, resulting in an enzyme which has approximately one-third of the molecular mass of the parent zymogen. More detailed investigations of the chemical properties of procarboxypeptidase A-S6 (Yamasaki *et al.*, 1963) have suggested that the zymogen is, in fact, an aggregate of three subunits, thus allowing for the possibility that each of the two enzymatic activities, the endopeptidase and carboxypeptidase A, is associated with a different subunit. After many fruitless attempts, it has in fact become possible to dissociate procarboxypeptidase A-S6 into three different fractions by use of concentrated urea or in aqueous solutions at *p*H 10.5. The products of disaggregation were separated from each other and the resulting fractions characterized chemically and enzymatically. The present report expands and supplements a preliminary announcement of these findings (Brown *et al.*, 1961).

In order to facilitate subsequent discussion, the terms "subunit" and "fraction" as used herein require definition. "Subunit" denotes the protein components which constitute the macromolecular complex of procarboxypeptidase A. The present concept proposes that individual protein subunits are responsible for the different enzymatic properties associated with this zymogen. Thus subunit I is defined as the protein moiety which is the zymogen of carboxypeptidase A. Analogously, subunit II is defined as the moiety which is the zymogen of the endopeptidase. The term "fraction" is defined operationally as the product of disaggregation. A fraction may be, but is not necessarily, identical with the corresponding subunit since alterations in structure such as denaturation or limited proteolysis may have occurred during the process used for disaggregation. For instance, as will be shown in this report, fraction I resembles carboxypeptidase A in chemical composition, but is devoid of enzymatic activity before or after treatment with trypsin. Although fraction II is the zymogen of the endopeptidase and can be activated with trypsin, some alterations in properties appear to have occurred during its isolation. A component of unknown function, subunit III, is postulated to be present in procarboxypeptidase A-S6, but absent in procarboxypeptidase A-S5 (Brown *et al.*, 1963).

MATERIALS AND METHODS

Procarboxypeptidase A-S6 was prepared as described in the preceding publication (Yamasaki *et al.*, 1963). Some of the experiments were carried out with the preparation isolated essentially as described by Keller *et al.* (1956, 1958), involving the combination of ammonium sulfate fractionation, isoelectric precipitation, and chromatography on DEAE-cellulose. No difference in properties was observed between materials prepared by either of these two methods.

Carboxypeptidase A was prepared from procarboxypeptidase A by a method to be described elsewhere (Cox *et al.*, 1963). Trypsin was a twice-crystallized preparation containing 50% $MgSO_4$, obtained from the Worthington Biochemical Corporation. Urea was a reagent grade preparation (Merck) and was recrystallized before use from 70% ethanol. All salts and other chemicals were reagent grade products. Sephadex G-25, lot TO 4650 was obtained from Pharmacia,

* Procarboxypeptidase A is the general term for zymogen preparations which, after tryptic activation, yield carboxypeptidase A. Procarboxypeptidase A-S6 denotes the protein having a sedimentation coefficient of 6 Svedberg units. This zymogen was previously described by Keller *et al.* (1956, 1958) and is also the subject of the present report. (see also Yamasaki *et al.*, 1963 [this issue]). Procarboxypeptidase A-S5 is a new form of procarboxy-peptidase A, characterized by a sedimentation coefficient of 5 Svedberg units. The preparation and properties of that zymogen are described in an accompanying report (Brown *et al.*, 1963).
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Uppsala, Sweden and, before use, was washed with 0.1 N NaOH, 95% ethanol, and finally with water. DFP^{32} was obtained from Volk Radio-Chemical Company, Chicago.[1] The manufacturer indicated a specific activity of 200 mc/g. At the time of the experiment, the radioactivity was 4.22×10^6 cpm per micromole of P_i.

The methods of amino acid analysis, amino terminal analysis, sedimentation analysis in the ultracentrifuge, and free-boundary electrophoresis were the same as described in the accompanying report (Yamasaki *et al.*, 1963). The *p*H was measured with a glass electrode using a Radiometer Type PHM 22p *p*H meter. Radioactivity measurements were performed in a Model 183 scaling unit and Model C110B counting unit equipped with a C11B printing timer, all made by Nuclear and Chemical Corporation, Chicago, Illinois. In plating DFP^{32} solutions, it was necessary to hydrolyze the DFP to diisopropylphosphate by adding 5 μl of 1 N NaOH to the sample on the planchette prior to drying.

The ratio of tyrosine-tryptophan was estimated spectrophotometrically from absorbancies at 294 mμ of the protein solutions in 0.2 NaOH and 0.1 N HCl (Brown, 1963) by use of the following equation:

$$\text{tyrosine-tryptophan} = (A_b - A_a)/A_a$$

where A_b is the absorbancy in 0.2 N NaOH and A_a the absorbancy in 0.1 N HCl, both measured at 294 mμ. The wavelength of 294 mμ was selected empirically from the absorption spectra of glucagon and lysozyme on the basis of tyrosine-tryptophan ratios of 2.00 and 0.50, respectively (Brown, 1963). These measurements were made in a Beckman Model DK-1 recording spectrophotometer.

The tyrosine-tryptophan ratio for fraction I was determined by the method of Bencze and Schmidt (1957), since fraction I is insoluble in 0.1 N HCl and thus could not be analyzed by the method of Brown (1963). In general, the two methods gave good agreement for proteins having a tyrosine-tryptophan ratio greater than one, but the method of Bencze and Schmidt appears to give values too low for proteins having tyrosine-tryptophan ratios of less than one.

Protein concentration was determined spectrophotometrically at 280 mμ using a value of $E_{280}^{0.1\%} = 1.9$ for the extinction coefficient of procarboxypeptidase (Keller *et al.*, 1956). Since the tyrosine and tryptophan contents of the products of molecular disaggregation differ from that of procarboxypeptidase A-S6, the use of the same extinction coefficient for all the proteins investigated in this study introduces systematic errors in the determination of protein concentrations. Since precise values for the extinction coefficients of the fractions were not available, these errors had to be ignored.

Electrophoresis in Sephadex Gel.—The use of Sephadex as a supporting medium for zone electrophoresis has not been previously reported. The resolution by this technique is similar to that of electrophoresis on a starch grain block. However, Sephadex has the advantage of being insoluble, and relatively stable to attack by microorganisms.

In order to prepare the gel blocks, Sephadex G-25 (25 g) is washed several times with 500 ml of 0.1 N NaOH followed by ethanol and water. The washed slurry is poured into a column (2 × 25 cm) and equilibrated by passing about 3 volumes of the desired buffer (0.05 M Veronal, *p*H 8.6) through the column. The equilibrated gel is then transferred from the column to a flask, enough buffer is added to make a thick slurry, and entrained air bubbles are removed by evacuation for about 1 minute. The slurry is then poured into a lucite tray (2 cm wide, 24 cm long, 0.6 cm deep) and the gel smoothed out with a celluloid X-ray film from which the emulsion was removed. The excess buffer is removed by blotting with filter paper at both ends.

The Spinco paper electrophoresis system is easily adapted for the Sephadex block procedure. The gel block is mounted above the center partition of the Durrum cell, and a filter-paper bridge is made at both ends of the block to the electrode vessels, by use of a double layer of filter-paper wick approximately 3 cm wide. The sample is applied to one or several pieces of filter paper which are cut to the dimensions of the cross section of the block and inserted vertically into the block. The gel is then carefully smoothed around the sample with a piece of celluloid. Alternatively, a celluloid dam of appropriate size is inserted into the block and the gel inside scooped out. The sample is then applied to an equal volume of dry Sephadex and the resulting paste is carefully packed inside the dam. In general, experiments are run at 200 v and 10 ma. Under these conditions, no cooling is necessary, and electrophoresis may be carried out at room temperature or in the cold room.

The location of the zones in the gel is simply determined by inserting a strip of filter paper on one edge to a depth of about 1 mm perpendicular to the surface, down the length of the block. The solution rises into the paper by capillary action and, when the solution front reaches the upper edge, the strip is removed, dried in an oven at 120° for 30 minutes, and stained with bromophenol blue. Electrophoresis may be resumed and, if desired, additional prints may be taken at a later time. Besides its simplicity, this technique has the advantage that the print comes from the entire volume of the block, rather than just from the surface, as in the blotting technique. An illustration of the experimental procedure is shown in Figure 1.

To elute the zones from the gel, the block is cut into appropriate sections as indicated from the filter-paper print. Each section is transferred to a fritted glass filter, layered with a small volume of buffer, and the eluate is forced through the filter with air pressure.

Enzymatic Activities.—Enzymatic assays were made using the synthetic substrate HPLA for carboxypeptidase A and ATEE for endopeptidase activity. In addition, a preparation of glucagon, obtained through the courtesy of Dr. Otto K. Behrens of the Lilly Research Laboratories, Eli Lilly and Company, was used for preliminary tests for the specificity of the endopeptidase. The assays involving the synthetic

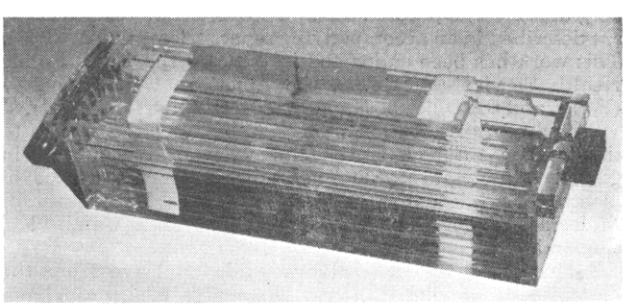

FIG. 1.—Experimental arrangement for zone electrophoresis in Sephadex gel. The vertical guide strip for the location of the zones is shown along the back of the trough. For further details see the text.

[1] The following abbreviations will be used: DFP = diisopropylphosphorofluoridate; DIP = diisopropylphosphoryl; HPLA = hippuryl-D,L-phenyllactic acid; ATEE = acetyl-L-tyrosine ethyl ester; FDNB = 1-fluoro-2,4-dinitrobenzene.

substrates HPLA and ATEE have already been described (Yamasaki *et al.*, 1963). Activities are expressed as the uptake of OH^- in μeq per min per mg protein (extinction coefficient $E^{0.1\%}_{280} = 1.9$).

Results

The first indication for the molecular disaggregation of procarboxypeptidase A resulted from a simple experiment involving paper electrophoresis of the zymogen in a buffer containing 8 M urea. In this experiment, the protein was dissolved in a 0.075 M sodium Veronal buffer, *p*H 8.6, containing 8 M urea, and subjected to electrophoresis in a Spinco Model RB paper electrophoresis apparatus for 12 hours at room temperature. After staining with bromophenol blue, two bands were observed. Densitometric analysis in the Spinco RB Analytrol indicated that the areas under the fast and slow components were in a ratio of 2.8:1.6. If the molecular weight of procarboxypeptidase A-S6 is taken as 87,000, and the sum of the areas of the two zones is assumed to be proportional to the molecular weight, then the areas of the two zones would be proportional to molecular weights of approximately 32,000 and 55,000, a result to be expected if the smaller and slower moving zone corresponded to a fraction having approximately the molecular weight of carboxypeptidase A and the faster, larger zone corresponding to the remainder of the zymogen molecule.

Separation of the Active Centers of Endopeptidase and Carboxypeptidase A

Previous work by Keller *et al.* (1956) had suggested that during the conversion of procarboxypeptidase A to carboxypeptidase, the endopeptidase which transiently appears during activation becomes disintegrated into peptide fragments. Since the endopeptidase is inhibited by DFP, an experiment was designed to determine in the following manner the fate of the active center of the endopeptidase.

Procarboxypeptidase A-S6 was first activated to the endopeptidase stage, the endopeptidase was labeled and inactivated with DFP^{32}, and then conversion to carboxypeptidase A was completed by incubation at 37° with additional amounts of trypsin and endopeptidase, the latter in the form of procarboxypeptidase A-S6. Under these conditions, carboxypeptidase A, which is formed, is derived from the DIP^{32}- protein as well as from the freshly added procarboxypeptidase A-S6. The activation mixture was then fractionated and the distribution of carboxypeptidase A activity and radioactive phosphorus among the protein fractions was determined.

In a typical experiment, 500 mg of procarboxypeptidase A-S6 was dissolved in 50 ml of an 0.01 M sodium phosphate buffer, *p*H 8, containing 1×10^{-5} M $ZnCl_2$ at 0°. After standing for one hour, the slightly turbid solution (9.47 mg protein per ml) was filtered through a fritted glass filter and the maximum endopeptidase and carboxypeptidase A activities determined after activation of small aliquots, yielding 102% and 70% of maximum activity, respectively. In order to saturate any nonspecific DFP binding sites, 0.05 ml of 1 M DFP was added to the zymogen solution and excess DFP removed by dialysis overnight, in the cold room, in a rocking dialyzer. The solution was next activated to the endopeptidase stage by the addition of 0.46 mg of trypsin followed by incubation for 10 hours at 0°. The endopeptidase was then labeled and inactivated by the addition of 36.6 μmoles of DFP^{32}, the excess reagent was removed by dialysis in the rocking dialyzer, and the DIP^{32}-protein was frozen until used for further experiments. Determination of the stoichiometry of the labeling indicated that 0.59 atom of phosphorus had been introduced per molecule of procarboxypeptidase A-S6. For complete conversion to carboxypeptidase A, 66.3 mg of DIP^{32} protein was incubated for 12 hours at 37° in a solution containing also 20 mg of procarboxypetidase A-S6 and 20 mg of trypsin. Assay of the activation mixture with HPLA indicated that approximately 80% of conversion had taken place. The solution was then dialyzed against 0.01 M potassium phosphate buffer, *p*H 7.8. Sixty-five per cent of the material absorbing at 280 mμ escaped from the dialysis bag, whereas 81% of the radioactivity and 85% of the carboxypeptidase activity were retained within the bag. When the activation mixture was precipitated with 15% trichloroacetic acid, 50% of the absorbancy at 280 mμ remained in the supernatant solution, whereas 91% of the radioactivity remained in the precipitate, thus giving further evidence that the radioactive label was attached to a larger protein fragment.

Zone electrophoresis of the activation mixture on paper, using the Spinco Model RB electrophoresis apparatus, resolved two protein bands as determined by staining with bromophenol blue. One band near the origin had a mobility similar to that of carboxypeptidase A used as a control, but had no radioactivity. Another band migrating rapidly toward the anode contained all of the radioactivity as determined by radioautography.

In order to prepare these fractions in sufficient amounts to allow measurement of carboxypeptidase A activity, protein, and radioactivity, the activation mixture was subjected to zone electrophoresis on a Sephadex gel block. The two protein zones, shown in Figure 2, were located by means of a filter-paper detection strip (see under Methods) and stained with bromophenol blue. The gel block was then cut into six sections, each packed in a glass filter (1 cm diameter) and the contents eluted by passing 2 ml of buffer through the filter cake. Analysis of the filtrate from the six sections is given in Figure 2. The results clearly show that all the carboxypeptidase activity was located in the zone near the origin, and that all the radioactivity was in the zone migrating toward the anode. The results provide unequivocal proof that the active center of the endopeptidase is associated with a protein fraction different from that which is responsible for carboxypeptidase A activity.

Sedimentation Analysis in Urea

More direct evidence for the molecular disaggregation in 8 M urea was obtained by sedimentation analysis

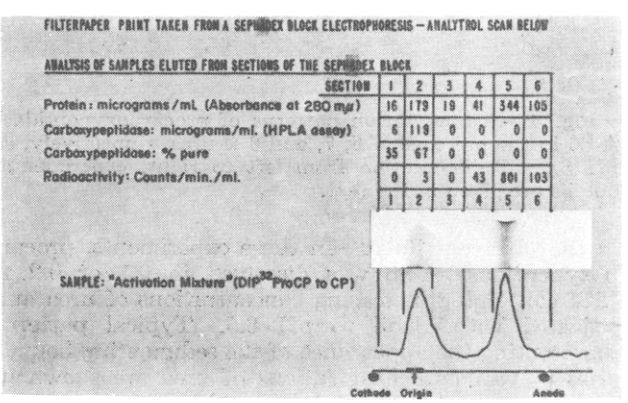

FIG. 2.—Zone electrophoresis on Sephadex gel of products of activation of DIP^{32} procarboxypeptidase A-S6. Conditions of electrophoresis were as follows: 200 v, 10–13 ma, room temperature, for 7.5 hours. Sodium Veronal buffer 0.075 M, *p*H 8.6. For further details, see the text.

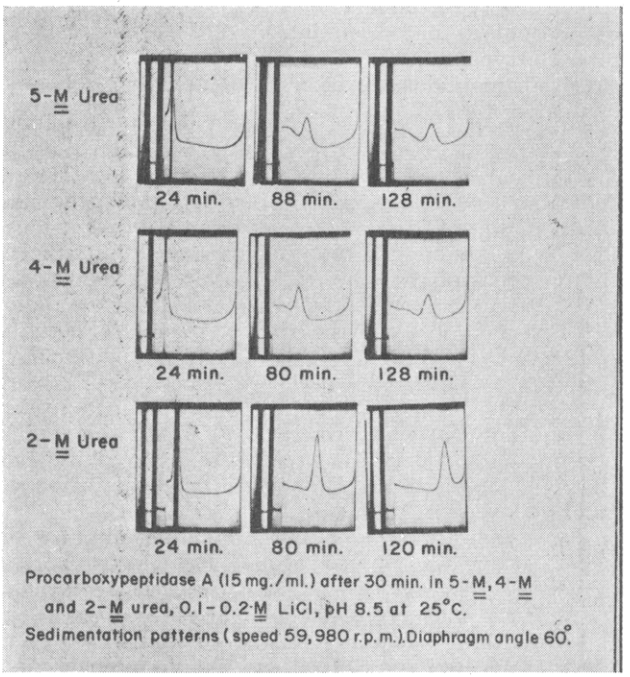

FIG. 3.—Sedimentation patterns of procarboxypeptidase A-S6, in the presence of 2, 4, and 5 M urea, respectively, at *p*H 8.5. Sedimentation from left to right. Experimental details are given in the text.

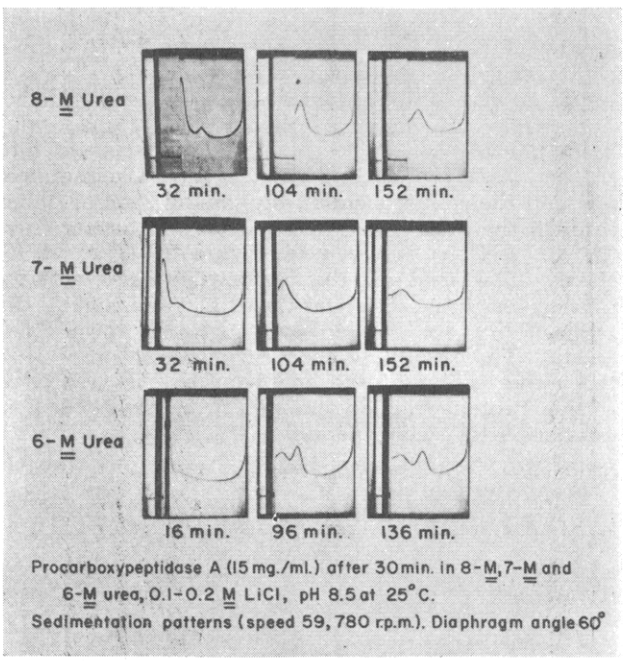

Fig. 4.—Sedimentation patterns of procarboxypeptidase A-S6 in the presence of 6, 7, and 8 M urea, respectively, at *p*H 8.5. Sedimentation from left to right, experimental details are given in the text.

in the ultracentrifuge. In these experiments, procarboxypeptidase A-S6 was dissolved in 0.1 or 0.2 M LiCl containing increasing concentrations of urea and adjusted with alkali to *p*H 8.5. Typical patterns representing the appearance of the sedimenting boundaries in various concentrations of urea are shown in Figures 3 and 4. It will be seen that up to 4 M urea exerted little effect on the sedimentation pattern; however, between 5 and 8 M urea, the initial boundary characteristic of procarboxypeptidase A-S6 was progressively replaced by a slower sedimenting component. In 6 M urea, nearly equal amounts of the two fractions are observed, whereas in 8 M urea the transformation to the slower moving component was complete. While calculation of sedimentation coefficients in the presence of such high concentrations of urea are uncertain, because of the large correction ofr the density and viscosity of the solvent, preliminary calculations indicate that the absolute value of the sedimentation coefficient of the slower moving component is of the order of that of carboxypeptidase A. This transformation to a slower sedimenting form is interpreted as due to disaggregation rather than due to an increase in hydrodynamic volume, since no change in the intrinsic viscosity of procarboxypeptidase in urea solutions was observed.

Alkaline Disaggregation

Alkaline buffers of *p*H 10.5 were found to be effective in producing disaggregation, leading to fractions which could be separated from each other by chromatography on DEAE-cellulose. Since slow activation of endopeptidase occurred, the addition of DFP to the disaggregation solution is essential in this case. Disaggregation at *p*H 10.5 was found to be time dependent, being completed after approximately 24 hours at 22°.

The disaggregation solution was prepared by dissolving procarboxypeptidase A-S6 in a *p*H 10.5 buffer containing 0.28 M LiCl, 0.1 M glycine, and 0.01 M DFP. The latter was added as a 1 M solution in isopropanol just prior to the addition of the protein, since in alkaline buffers the life of DFP is relatively short. The disaggregation solution was prepared at 0° and was kept at that temperature for at least one hour before exposing it to room temperature.

Sedimentation Analysis.—The course of disaggregation is shown in Figure 5. After 9 hours at *p*H 10.5, the sedimenting boundary revealed two peaks of comparable area, indicating that approximately 50% disaggregation had occurred. Sedimentation coefficients (uncorrected) of the two boundaries were, respectively, s'_{20} = 4.6 S, and 2.5 S. After 24 hours of disaggregation, the sedimentation pattern shows approximately 90% conversion of the faster to the slower peak (Fig. 5).

Fractionation of Disaggregation Mixture

During disaggregation at *p*H 10.5, a protein precipitate formed spontaneously in some instances, whereas in others precipitation occurred when the disaggregation mixture was dialyzed against 0.005 M phosphate buffer, *p*H 8, in preparation for subsequent chromatog-

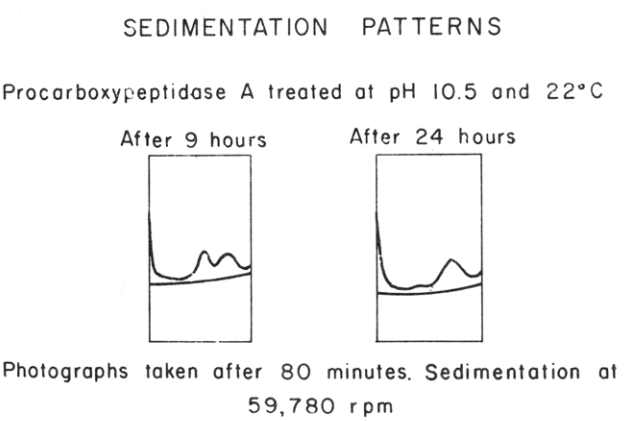

FIG. 5.—Sedimentation patterns of procarboxypeptidase A-S6 treated at *p*H 10.5, 22°, for 9 and 24 hours, respectively. Protein concentration 17 mg/ml. Sedimentation from right to left.

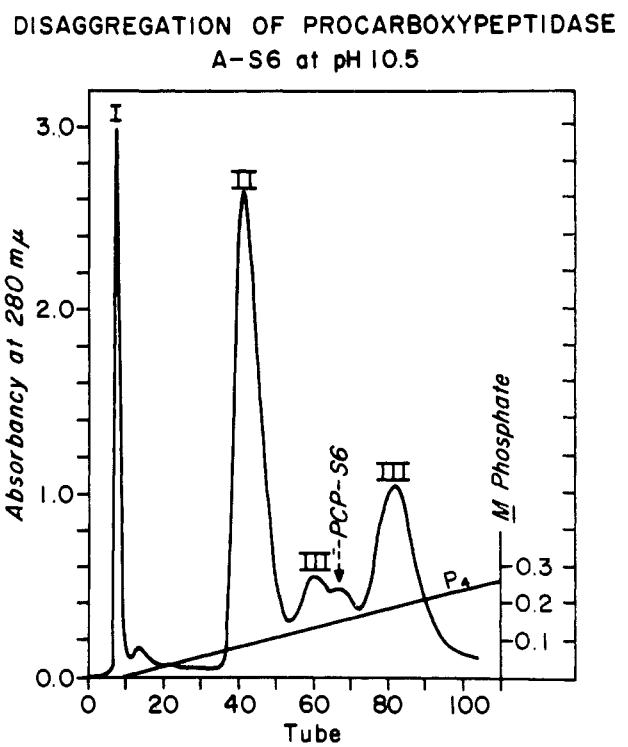

FIG. 6.—Chromatographic separation on DEAE-cellulose at *p*H 8.0 of the products of disaggregation of procarboxypeptidase A-S6 (preparation "JRB 20") at *p*H 10.5. The major peaks, in order of elution, represent fractions I, II, and III, the minor peaks represent fraction III′ and residual procarboxypeptidase.

raphy on DEAE-cellulose. Chromatography of the soluble proteins resolved the fractions of the disaggregation mixture as shown in Figure 6. Fraction I happened to remain soluble in this particular experiment and emerged with the breakthrough fraction (and precipitated in the collecting tubes), whereas in other instances it appeared as a precipitate. In addition to the other two major fractions, II and III, Figure 6 reveals a minor fraction, III′ which is believed to be a derivative of fraction III (see below), and a shoulder representing residual procarboxypeptidase A-S6. The zymogen preparation which was used in this experiment contained in addition to the usual three amino terminal groups, one-third equivalent of amino terminal threonine (preparation "JRB 20" [Brown *et al.*, 1963]). When a preparation was used which contained one full additional equivalent of amino terminal threonine (preparation "MY 24"), the chromatogram of the disaggregation mixture revealed that fraction III was entirely replaced by fraction III′ (Fig. 7), suggesting that these two fractions are interrelated. The significance of these findings will be considered in connection with the individual fractions. The effluents corresponding to fractions I, II, and III (or III′) were collected and after lyophilization the isolated fractions were used for further characterization.

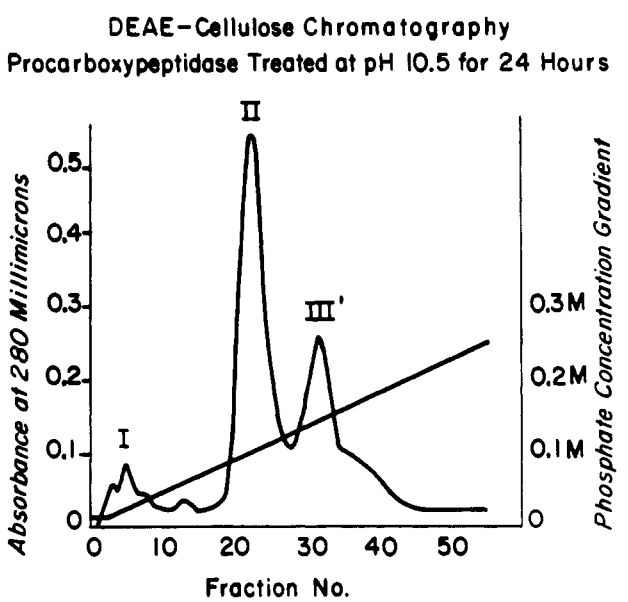

FIG. 7.—Chromatographic separation on DEAE-cellulose at *p*H 8.0 of the products of disaggregation of another preparation of procarboxypeptidase A-S6, at *p*H 10.5. This preparation ("MY 24") contained, in addition to the usual end groups, one equivalent of threonine as amino terminal group, and fraction III was entirely replaced by fraction III′ (see Fig. 6 and the text.)

Properties of the Isolated Fractions

Fraction I.—Fraction I was found to be insoluble in aqueous buffers of *p*H 6–8 containing up to 1 M NaCl. It was also insoluble in urea solutions up to 8 M containing 0.1 M NaCl. However, it was soluble in anhydrous formic acid, or in 0.1 N NaOH. Fraction I has thus the properties of a denatured protein, and it was therefore not surprising that no carboxypeptidase A or endopeptidase activity could be demonstrated for this fraction either before or after the attempted activation with trypsin.

Fraction I resembles, however, carboxypeptidase A in its amino acid composition. Table I gives the results of individual analyses and Table II gives the composition after appropriate corrections. For comparison, the amino acid composition of bovine pancreatic carboxypeptidase A is also given in Table II (Bargetzi *et al.*, 1963).

Calculation of the tyrosine-tryptophan ratio according to the method of Bencze and Schmidt (1957) gives a value of 2.35, which is in close agreement with

TABLE I

AMINO ACID ANALYSIS OF FRACTION I[a]

	Hours of Hydrolysis			
	22	48	86	18[b] (oxidized)
Lysine	15.3	13.7	15.2	13.6
Histidine	7.50	6.91	7.60	6.40
Arginine	12.9	11.3	12.5	11.4
Aspartic acid	27.9	27.1	27.3	28.4
Threonine	23.6	22.4	21.9	23.8
Serine	30.8	27.7	25.2	30.4
Glutamic acid	28.4	27.9	27.7	27.9
Proline	10.9	11.4	11.5	11.3
Glycine	25.7	25.2	25.0	25.2
Alanine	20.0	19.9	20.1	20.0
Half-cystine	1.70	1.64	Trace	2.49
Valine	15.0	15.6	15.9	16.3
Methionine	2.80	2.59	2.90	2.86
Isoleucine	18.3	20.0	21.8	17.0
Leucine	24.4	23.6	23.9	24.0
Tyrosine	18.5	18.2	18.3	
Phenylalanine	16.4	15.9	16.2	16.0

[a] The data are expressed as amino acid residues per molecule assuming a molecular weight of approximately 34,500.
[b] These data were obtained from a performic-acid-oxidized sample of fraction I. Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively. No corrections were applied for losses during performic acid oxidation.

TABLE II

CORRECTED AMINO ACID COMPOSITION OF FRACTION I[a] AND OF CARBOXYPEPTIDASE A

Amino Acid	Fraction I	Carboxypeptidase A	Amino Acid	Fraction I	Carboxypeptidase A
Lysine	14.7	15	Alanine	20.0	20
Histidine	7.34	8	Cysteic acid	2.49[b]	2
Arginine	12.2	11	Valine	15.9	16
Aspartic acid	27.4	28	Methionine	2.86	3
Threonine	24.2	27	Isoleucine	21.8	20
Serine	33.2	33	Leucine	24.0	23
Glutamic acid	28.0	25	Tyrosine	18.3	19
Proline	11.3	10	Phenylalanine	16.2	16
Glycine	25.3	22.5	Tryptophan	7.8[c]	8

[a] The data are expressed as residues per molecule, assuming a molecular weight of approximately 34,500. The composition represents the mean of the data given in Table I. Threonine, serine, valine, and isoleucine were corrected for hydrolysis time. The data for carboxypeptidase A are those of Bargetzi *et al.* (1963). [b] Half-cystine was determined as cysteic acid in the hydrolyzate of fraction I which was oxidized with performic acid. [c] Tryptophan was estimated from the tyrosine-tryptophan ratio of 2.34 which was determined spectrophotometrically from the absorption spectrum of fraction I in 0.2 N NaOH according to the method of Bencze and Schmidt (1957).

the value of 2.45 recently determined for carboxypeptidase A by Bargetzi *et al.* (1963) by the same technique, thus lending further support to the belief that fraction I is derived from that subunit of procarboxypeptidase A-S6, which gives rise to carboxypeptidase A activity. The amino acid composition of fraction I is nearly identical with that of carboxypeptidase A (Table II).

Table III presents the amino terminal groups found in preparations of fraction I by use of the Sanger FDNB technique. The results are expressed as residues per molecule, assuming a molecular weight of approximately 34,500. No corrections were applied to the data for losses during analysis. The data indicate that aspartic acid or asparagine is the principal amino terminal group.

Since aspartic acid (or asparagine) is one of the amino terminal groups of procarboxypeptidase A-S6, and also the amino terminal group of carboxypeptidase A (Coombs and Omote, 1962), it was suggested in the preliminary report of these data (Brown *et al.*, 1961) that the same aspartic acid (or asparagine) residue was involved in each case. It will be shown in the following paper, however, that this conclusion is not valid, and that subunit I, in contrast to fraction I, must contain lysine as the amino terminal group. The significance of these findings will be discussed in the accompanying paper (Brown *et al.*, 1963).

Fraction II.—Fraction II is a homogeneous protein as judged by sedimentation analysis (Fig. 8). Measurements of the dependence of the sedimentation coefficient on protein concentration, performed in a 0.04 M potassium phosphate buffer, *p*H 6.5, yielded on extrapolation to zero protein concentration a value of $s_{20^\circ,w} = 2.88$ S.

Enzymatic Properties.—Fraction II was devoid of enzymatic activity toward the substrates ATEE or HPLA. However, when fraction II was incubated with trypsin, endopeptidase activity rapidly appeared (ATEE as substrate) but no activity toward HPLA was found. The time course of activation is shown in Figure 9. Maximum activity corresponded to approximately 127 μeq OH^-/min/mg protein. In addition to ATEE, activated fraction II also hydro-

TABLE III

AMINO END-GROUP ANALYSIS OF FRACTION I

DNP-Amino Acid	Residues per Molecule[a]
Aspartic acid	0.46
di-Lysine	0.15
Alanine	0.14
Serine	0.10
Threonine	0.09

[a] Calculated on the basis of a molecular weight of 34,500 with no correction for losses.

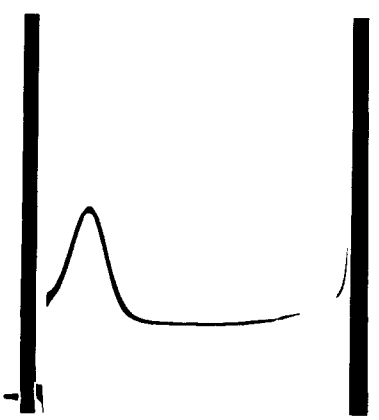

FIG. 8.—Sedimentation pattern of fraction II (1.1% protein) in 0.1 M potassium phosphate buffer, *p*H 8.0. Sedimentation for 60 minutes at 59,780 rpm, bar angle 60°.

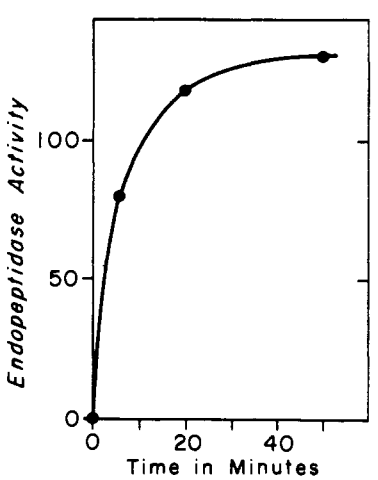

FIG. 9.—Time course of activation at 0° of fraction II. Conditions of activation were as follows: Fraction II, 0.447 mg/ml; trypsin, 0.010 mg/ml; 0.04 M potassium phosphate buffer, containing 0.1 M NaCl, *p*H 6.5. Substrate is ATEE. Activity is expressed as uptake of OH^- in μeq per min per mg protein.

TABLE IV

AMINO ACID ANALYSIS OF FRACTION II[a]

Preparation:	26	26	26	26	39
Hours of Hydrolysis:	20.5	92	18	16	48
Remarks:			Oxidized[b]	DNP-Oxidized[c]	
Lysine	7.21	7.35	7.57	...	8.03
Histidine	4.76	4.94	4.99	...	5.48
Arginine	8.57	8.90	8.13	7.20	8.79
Aspartic acid	25.3	26.2	27.1	27.6	25.0
Threonine	16.5	16.2	16.5	16.7	16.0
Serine	13.6	11.7	13.8	13.0	12.9
Glutamic acid	22.6	22.9	22.5	22.6	23.0
Proline	11.9	11.7	11.2	12.0	11.9
Glycine	22.7	22.9	22.8	23.0	22.8
Alanine	16.3	16.1	16.2	16.0	16.2
Half-cystine	6.69	6.82	8.89	7.83	8.86
Valine	16.2	19.6	15.4	16.1	18.4
Methionine	1.09	0.78	0.97	1.04	1.07
Isoleucine	11.3	13.5	11.2	11.7	13.1
Leucine	21.1	21.6	21.1	21.0	21.3
Tyrosine	6.73	7.43	...	...	6.75
Phenylalanine	6.48	7.96	6.20	7.83	7.01

[a] The data are expressed as residues per molecule, assuming a molecular weight of approximately 27,000. [b] This sample was oxidized with performic acid. Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively. [c] Analysis of hydrolyzate of oxidized DNP-fraction II.

TABLE V

CORRECTED AMINO ACID COMPOSITION OF FRACTION II[a]

Amino Acid		Amino Acid	
Lysine	7.53	Alanine	16.2
Histidine	5.06	Cysteic acid	8.89[b]
Arginine	8.75	Valine	19.6
Aspartic acid	25.5	Methionine	0.98
Threonine	16.8	Isoleucine	13.5
Serine	14.3	Leucine	21.3
Glutamic acid	22.8	Tyrosine	6.97
Proline	11.8	Phenylalanine	7.15
Glycine	22.8	Tryptophan	13.9[c]

[a] The data are expressed as residues per molecule, assuming a molecular weight of 27,000. The composition represents the mean of the data given in Table IV for the 20.5-, 48-, and 92-hour hydrolyzates. Threonine, serine, valine, and isoleucine were corrected for time of hydrolysis. [b] Half-cystine was determined as cysteic acid in hydrolyzate of fraction II which was oxidized with performic acid. The value given is uncorrected for losses during oxidation. Application of the correction factor of Hirs (1956) yields 10 residues per molecule. [c] Tryptophan was estimated from the tyrosine-tryptophan ratio of 0.50 which was determined spectrophotometrically at 294 mμ (Brown, 1963).

lyzed acetyl-L-tryptophan ethyl ester, *p*-nitrophenyl-acetate, casein, and glucagon. Activated fraction II had no proteinase activity toward elastin and no esterase activity toward methyl butyrate.

Amino Acid Composition.—The results of amino acid analysis of fraction II are given in Table IV and Table V.

Amino Terminal Groups.—When fraction II was reacted with FDNB and subsequently hydrolyzed, no significant quantities of ether-soluble DNP-amino acids were found. When the DNP-protein was oxidized with performic acid and then hydrolyzed for 16 hours in constant-boiling HCl, DNP-cysteic acid was found in the acid-soluble phase. The recovery without corrections for losses was 0.35 residues per molecule (molecular weight 27,000). The ether-soluble phase again contained no significant quantities of DNP-amino acids.

Amino acid analysis of the acid hydrolyzate of the oxidized DNP-protein gave 7.8 residues of cysteic acid per molecule (Table IV), whereas performic-acid-oxidized fraction II itself gave 8.9 residues of cysteic acid per molecule. The difference, 1.1 residues, corresponds to the stoichiometric loss of one half-cystine per molecule in the DNP-protein, a finding entirely consistent with the assumption that half-cystine occupies the amino-terminal position in fraction II. In this respect, as well as in enzymatic specificity, fraction II resembles closely bovine pancreatic chymotrypsins A and B.

Mechanism of Activation.—Since fraction II is the zymogen of a chymotrypsinlike endopeptidase, it was of particular interest to examine the mechanism of activation of this fraction. Because of the limited amounts of material available, however, this study had to be restricted to end-group analysis.

Activation by trypsin was carried out at 0°, and when maximum activity toward ATEE was approached DFP was added to inhibit both the endopeptidase and trypsin. Inactivation was allowed to proceed for 3 hours at 0°. The solution was then dinitrophenylated and analyzed for DNP end groups by the usual procedure. Two-dimensional chromatography of the ether-soluble DNP-amino acids yielded only two spots of quantitative significance. One of these was identified as DNP-valine, whereas the other spot did not correspond to any known DNP-amino acids, and thus was tentatively identified as a DNP-peptide. In addition, the chromatogram also revealed a spot of di-DNP-cystine, thus indicating that the amino terminal half-cystine of fraction II was still present in the activated protein (Fig. 10).

The spots corresponding to DNP-valine and DNP-peptide were eluted with 1% sodium bicarbonate and

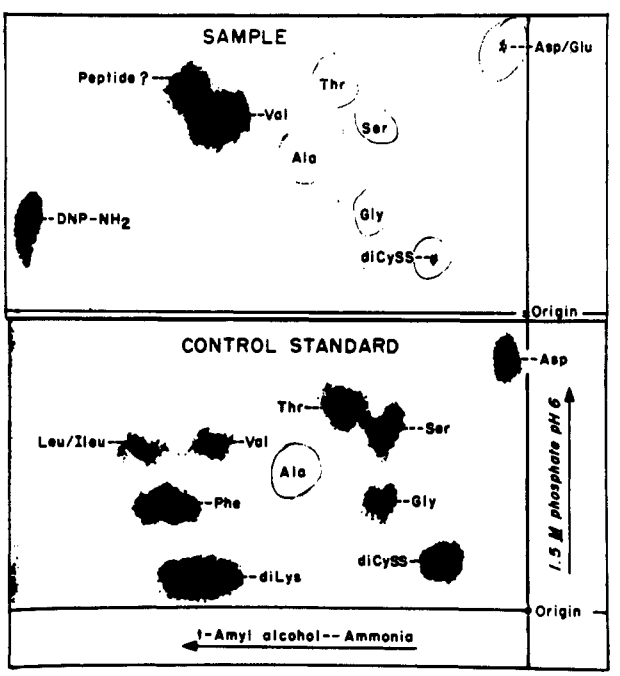

FIG. 10.—Ether-soluble DNP end groups of activated fraction II. The upper chromatogram reveals DNP-valine and a DNP-peptide as the major products, and the following DNP-amino acids as trace impurities: threonine, serine, alanine, glycine, and aspartic or glutamic acid. di-DNP-cystine represents the amino terminal group of fraction II, present before activation. The lower chromatogram shows the control DNP amino acids for comparison.

TABLE VI

AMINO ACID ANALYSIS OF FRACTIONS III AND III'[a]

Hours of Hydrolysis:	24	91	18	25	72	24	16
Fraction:	III'	III'	III'	III	III	III	III
Remarks:			Oxidized[a]			Oxidized[b]	DNP[c]
Lysine	8.19	7.64	6.66	—	7.50	7.51	—
Histidine	5.88	6.01	5.28	—	6.16	5.94	—
Arginine	6.72	7.03	5.94	—	7.20	7.51	7.16
Aspartic acid	20.5	20.8	22.2	22.5	21.3	23.6	21.4
Threonine	9.37	8.85	9.08	9.85	9.23	9.90	9.20
Serine	15.1	10.0	14.1	15.5	13.1	15.1	12.6
Glutamic acid	22.3	21.3	21.5	24.9	23.9	24.5	23.2
Proline	12.9	13.7	12.4	12.9	12.8	12.7	12.0
Glycine	20.2	19.7	19.8	19.9	19.8	20.1	19.7
Alanine	14.8	15.3	15.2	15.1	15.2	14.9	15.3
Half-cystine	5.08	6.01	6.08	6.70	6.30	5.79	5.88
Valine	15.3	18.9	15.6	16.6	19.7	17.1	15.7
Methionine	0.64	1.24	1.04	2.03	1.96	1.97	1.78
Isoleucine	9.43	11.3	9.86	10.9	11.2	9.93	9.97
Leucine	15.0	15.5	15.2	18.0	17.2	17.4	16.8
Tyrosine	4.71	5.00	—	5.51	5.31	—	—
Phenylalanine	—	6.82	6.14	7.26	7.07	7.13	6.64

[a] The data are expressed as amino acid residues per molecule. The molecular weight is taken as approximately 24,000. [b] In the performic acid oxidized samples, half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively. [c] The acid hydrolyzate of DNP-fraction III' was used for this analysis.

the spectra of the solutions recorded in a Beckman Model DK-1 spectrophotometer. The results are shown in Figure 11, which demonstrate a blue shift of the absorption maximum of the unknown compound of approximately 7 mμ, as compared to the DNP-amino acid, a phenomenon characteristic of DNP-peptides (Levy, 1955). The recoveries of DNP-valine and DNP-peptide, without any correction for losses, were 0.45 and 0.28 residues per molecule, respectively, assuming a molecular weight of 27,000, and an extinction coefficient at 360 mμ of 2.86. The results indicate that upon activation of fraction II, a new amino terminal group, valine, is formed on the protein. The present findings are similar to the events accompanying the conversion of chymotrypsinogen A to π-chymotrypsin (Bettelheim and Neurath, 1955), except

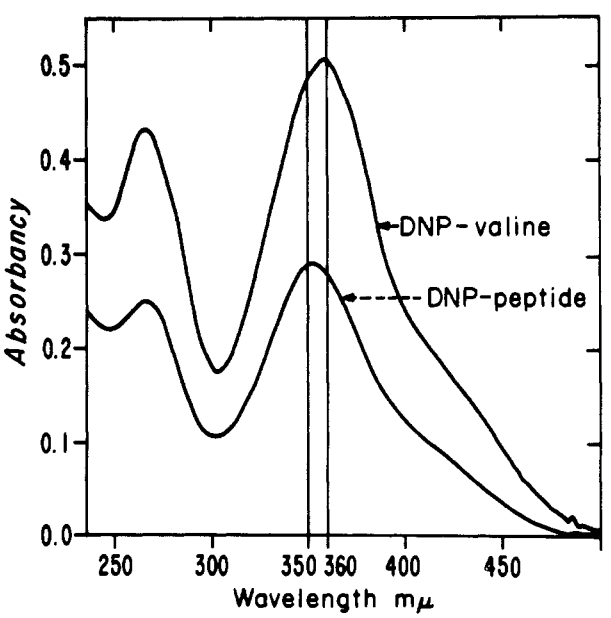

FIG. 11.—Comparison of absorption spectra of the two major ether-soluble DNP end groups of activated fraction II, DNP-valine and DNP-peptide (see Fig. 10 and the text). Spectra recorded in a Beckman DK-1 spectrophotometer, solvent: 1% $NaHCO_3$.

that the newly formed amino terminal group is valine instead of isoleucine.

Fraction III.—This fraction has been obtained in two forms, III and III' (see above), and since the latter is probably a product of partial degradation of the former, in most of the experiments both fractions were analyzed to determine their similarities and differences.

Sedimentation Analysis.—Fractions III and III', when dissolved in a 0.05 M phosphate buffer, *p*H 6.5, each gave a symmetrical boundary with a sedimentation coefficient of $s_{20,w}$ = 2.9 S (protein concentration 0.7%).

Amino Acid Analysis.—The results of amino acid analysis of fractions III and III' are given in Table VI. The relative compositions of the fractions, after proper corrections for losses or incomplete hydrolysis, are given in Table VII. Tryptophan was estimated from the tyrosine-tryptophane ratio of 0.51 (fraction III') determined spectrophotometrically. The data are expressed on the basis of an assumed molecular weight of 24,000, calculated by difference from the molecular weights of procarboxypeptidase A-S6 and of fractions I and II. Fractions III and III' resemble each other very closely in amino acid composition, leaving little doubt that these proteins are related. Calculated differences between them are also included in Table VII. In each case, it is fraction III' which is deficient in amino acids, consistent with the view that it presents a partially degraded form. Both fractions III and III' resemble fraction II in amino acid composition, but such differences as have been found (e.g., histidine, arginine, threonine, serine, half-cystine, methionine, tyrosine, and tryptophane) are outside the limits of experimental error and, therefore, are considered to be significant. Amino terminal analysis on fraction III yielded 0.3 residue per molecule of DNP-aspartic acid and 0.2 residue of di-DNP-lysine (uncorrected for losses).

Enzymatic Properties.—No enzymatic activity could be found for fraction III or fraction III', before or after activation with trypsin. The following substrates were tested: HPLA, ATEE, *p*-nitrophenylacetate, elastin, casein, glucagon, methylbutyrate, DNA, RNA, and uridine-3',5'-phosphate. Tests for amylase and lipase activity were also negative. Fraction III had

TABLE VII

CORRECTED AMINO ACID COMPOSITIONS OF FRACTION III AND FRACTION III'[a]

	Fraction III	Fraction III'	Difference[b]
Lysine	7.51	7.50	
Histidine	5.94	5.72	
Arginine	7.29	6.56	1
Aspartic acid	21.9	20.7	1
Threonine	10.2	9.6	1
Serine	17.1	17.1	
Glutamic acid	24.4	21.7	3
Proline	12.9	13.0	
Glycine	19.9	19.9	
Alanine	15.2	15.1	
Cysteic acid	5.9	6.1	
Valine	19.1	18.9	
Methionine	1.94	0.97	1
Isoleucine	11.3	11.2	
Leucine	17.4	15.2	2
Tyrosine	5.41	4.86	
Phenylalanine	7.03	6.82	
Tryptophan	10.6	9.5	1

[a] The data are expressed as amino acid residues per molecule, assuming a molecular weight of approximately 24,000. [b] Difference between fractions III and III' corrected to the nearest integer.

no inhibitory effect on the hydrolysis of HPLA by carboxypeptidase A. The physiological significance of subunit III thus remains a problem for future research. Lack of enzymatic activity may simply be due to the inappropriate choice of substrates that have been tested or due to the fact that fraction III, like fraction I, is inactivated by the isolation procedures.

DISCUSSION

The experimental data presented in this report provide evidence for the hypothesis that procarboxypeptidase A-S6 is a complex of three different protein subunits. The relationships of the subunits to the products of activation or disaggregation of procarboxypeptidase A-S6 are shown in Figure 12. A more comprehensive scheme including procarboxypeptidase A-S5 and its derivatives are given in the accompanying paper (Brown *et al.*, 1963).

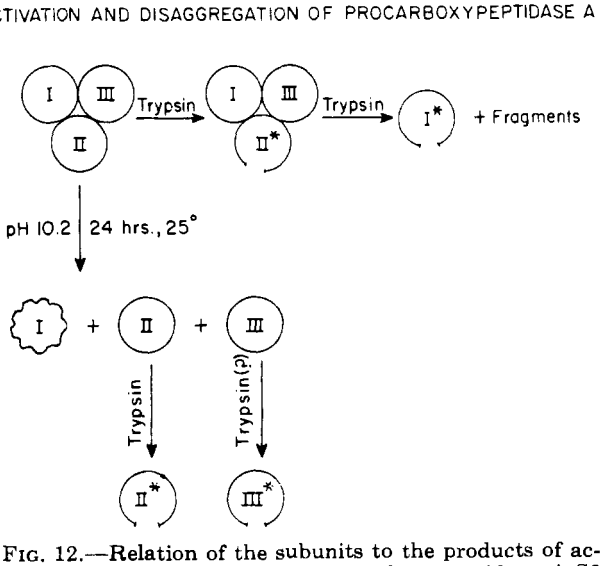

FIG. 12.—Relation of the subunits to the products of activation and disaggregation of procarboxypeptidase A-S6 (taken from Brown *et al.*, 1961).

The finding of three amino terminal groups in procarboxypeptidase A-S6, i.e., lysine, aspartic acid (or asparagine), and half-cystine suggests that the zymogen is composed of three polypeptide chains corresponding to each of these three terminal groups. Sedimentation analysis of solutions of procarboxypeptidase A-S6 in urea or in buffers at *p*H 10.5 show a transformation from a species characterized by a sedimentation coefficient of 6 S to one of 3 S, consistent with disaggregation into three fractions of approximately equal molecular weight. Since disaggregation was effected by reagents which normally do not cleave disulfide bonds, the subunits must be held together in the parent molecule by bonds other than covalent ones.

The successful isolation and characterization of the fractions from disaggregation mixtures provides most convincing evidence for the subunit structure of procarboxypeptidase A-S6. A difficult but important part of the present studies was the demonstration of differences and similarities between the properties of a subunit and its corresponding fraction.

Since subunit I has been defined as the zymogen of carboxypeptidase A, it must have a molecular weight equal to or greater than carboxypeptidase A, and it must contain each amino acid present in the carboxypeptidase A molecule. The similarity if not identity in amino acid composition of fraction I and carboxypeptidase A is evidence in support of this conclusion. Proof for the identity in enzymatic properties has to await conditions of disaggregation which leave fraction I in the native form. The relationship of the amino terminal groups of subunit I, fraction I, and carboxypeptidase A is a more complex problem. As will be shown in the following paper of this series (Brown *et al.*, 1963), there is reason to believe that the amino terminal group of subunit I is in fact lysine in contrast to asparagine found in fraction I or in certain preparations of carboxypeptidase A (Thompson, 1953; Coombs and Omote, 1962). However, the amino terminal group of fraction I is aspartic acid (or asparagine), indicating that the amino terminal region of subunit I is split off during alkaline disaggregation as well as in the zymogen-enzyme conversion. The reason for the degradation of subunit I during alkaline disaggregation is not known. High concentrations of DFP were used as a precaution against the formation of endopeptidase. Since fraction II was isolated from the alkaline disaggregation mixture with no amino terminal group present other than the half-cystine characteristic of subunit II, it would appear that inhibition by DFP was effective and complete. However, since fraction I is denatured, it may be particularly susceptible to traces of proteolytic activity. Thus an important problem for future research is to find conditions of disaggregation which leave fraction I in the undenatured form. Such studies are now in progress (Walsh, Kumar, Freisheim, and Neurath, unpublished).

Of the three fractions isolated from alkaline disaggregation mixtures, fraction II is the most interesting one, since its properties closely approximate those expected for subunit II. Fraction II appears to be the true zymogen of an endopeptidase. The presence of half-cystine as amino terminal group is consistent with the presence of the same amino terminal group in procarboxypeptidase A-S6 or S5. Fraction II appears to be akin in several respects to chymotrypsinogens A and B of the bovine pancreas. Each of these three proteins is the zymogen of an endopeptidase which hydrolyzes the ester substrate ATEE, and each enzyme is inhibited by DFP. Most strikingly, perhaps, each zymogen contains half-cystine as the amino terminal group. It should be emphasized, however,

TABLE VIII

COMPARISON OF AMINO ACID COMPOSITIONS OF FRACTION II AND CHYMOTRYPSINOGENS A AND B[a]

	Fraction II	Chymotrypsinogen A	Chymotrypsinogen B
Lysine	7	13	10
Histidine	5	2	2
Arginine	8	4	5
Aspartic acid	24	22	19
Threonine	16	23	20
Serine	13	30	19
Glutamic acid	21	14	16
Proline	11	9	12
Glycine	21	23	20
Alanine	15	22	21
Half-cystine	8	10	8
Valine	18	22	21
Methionine	1	2	4
Isoleucine	12	10	8
Leucine	20	19	17
Tyrosine	6	4	3
Phenylalanine	7	7	6
Tryptophan	13	7	6

[a] The data are expressed as residues per molecule, assuming molecular weights of 25,000 for fraction II and chymotrypsinogen A, and 24,000 for chymotrypsinogen B. The compositions for chymotrypsinogens A and B are based on data of Wilcox *et al.* (1957) and Kassell and Laskowski (1961), respectively.

that these three proteins are not the same, and are not derived from one another. This is best illustrated by comparing the amino acid composition of fraction II with that of chymotrypsinogens A and B as shown in Table VIII. Though a number of similarities may be noted among the compositions of these three zymogens, small differences appear to be the rule.

The relationships of fraction III and III′ to subunit III and the lack of enzymatic activity or other demonstrable physiological functions associated with subunit III have already been discussed in this report. The elucidation of the physiological function of subunit III is an important problem that remains to be solved.

If the fractions isolated from alkaline disaggregation mixtures do in fact correspond closely to the subunits of procarboxypeptidase A-S6, then the sum of the three fractions should equal the molecular weight and amino acid composition of the parent molecule. At the present, precise molecular weight data are available for procarboxypeptidase A-S6 (Yamasaki *et al.*, 1963), but not for the individual fractions. In a preliminary report of the present investigation (Brown *et al.*, 1961), molecular weights were calculated on the assumptions of 3, 1, and 2 methionine residues per molecule for fractions I, II, and III, respectively. On that assumption, the sum of the three fractions gave a molecular weight of 87,000 and an amino acid composition which were in excellent agreement with those to be expected from procarboxypeptidase A-S6. Subsequent experiments, however, have indicated 7 rather than 6 methionine residues per molecule of procarboxypeptidase A-S6. Comparison of the amino acid composition of the sum of the fractions with that of procarboxypeptidase A-S6, on the basis of the present data, is given in Table IX. On the whole, the present results are consistent with the hypothesis that procarboxypeptidase A-S6 is a complex of three subunits and that the three fractions isolated from alkaline disaggregation mixtures correspond closely to these subunits in amino acid composition and molecular weight.

TABLE IX

AMINO ACID COMPOSITION OF PROCARBOXYPEPTIDASE A-S6 AND FRACTIONS I, II, AND III[a]

	Assumed Molecular Weights				
	PCP-S6 87,000	I + II + III 87,000	I 36,000	II 27,000	III 24,000
Lysine	30	31	15	8	8
Histidine	19	19	8	5	6
Arginine	29	30	13	9	8
Aspartic acid	77	78	29	26	23
Threonine	49	52	25	17	10
Serine	62	67	35	14	18
Glutamic acid	78	77	29	23	25
Proline	39	37	12	12	13
Glycine	66	70	26	23	21
Alanine	53	53	21	16	16
Half-cystine	18	18	2	10	6
Valine	58	57	17	20	20
Methionine	7	6.0	3.0	1.0	2.0
Isoleucine	45	48	23	13	12
Leucine	65	64	25	21	18
Tyrosine	29	32	19	7	6
Phenylalanine	29	31	17	7	7
Tryptophan	29	33	8	14	11

[a] The compositions are expressed as residues per molecule.

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