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Bovine Pancreatic Procarboxypeptidase B. I. Isolation, Properties, and Activation*

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A chromatographic procedure has been developed for the isolation in pure form of bovine procarboxypeptidase B. The purified zymogen is homogeneous, as judged by chromatography, sedimentation analysis, moving boundary electrophoresis, and potential enzymatic activity. The activation of the zymogen is mediated by trypsin and presumably occurs in two steps. An initial fast reaction leads to the appearance of 60–70% of the maximal activity without any significant change in the sedimentation coefficient of the protein. The second step occurs more slowly and leads to the formation of a protein with a lower sedimentation coefficient and full enzymatic activity. Crystalline carboxypeptidase B has been isolated from activation mixtures. This enzyme is active toward the basic substrate benzoylglycyl-L-arginine but also hydrolyzes substrates for carboxypeptidase A, such as hippurylphenyllactic acid and carbobenzoxyglycyl-L-phenylalanine. Evidence is presented that the activities toward basic and aromatic compounds reside in the same enzyme.

Aqueous extracts of acetone powder of bovine pancreas contain zymogens for several of the proteolytic enzymes known to occur in the exocrine secretions of the gland. The most acidic of the zymogens, and hence the one which emerges last when such extracts are subjected to chromatography on DEAE-cellulose, is procarboxypeptidase A (Keller *et al.*, 1958b). This protein has recently been shown to be a complex of three subunits (Brown *et al.*, 1961) and the precursor of two different enzymes, carboxypeptidase A and an endopeptidase similar in specificity to chymotrypsin (Keller *et al.*, 1958a).

This protein, however, is not the only potential source of carboxypeptidase A activity in aqueous extracts of pancreatic acetone powder. Another fraction, much less strongly absorbed onto DEAE-cellulose, also gives rise, after tryptic activation, to activities against the ester hippurylphenyllactic acid (Snoke and Neurath, 1949) and the peptide carbobenzoxyglycyl-L-phenylalanine (Hofmann and Bergmann, 1940), which are typical substrates of carboxypeptidase A. However, this protein fraction, which is the subject of the present report, also shows potential activity against benzoylglycyl-L-arginine and thus possesses also the specificity characteristics of procarboxypeptidase B (Folk, 1956). This pancreatic procarboxypeptidase has been isolated in pure form and characterized, and a crystalline carboxypeptidase has been prepared from the purified zymogen after tryptic activation. Evidence has also been obtained that the active enzyme possesses in fact dual specificity and hydrolyzes substrates for both carboxypeptidases A and B. Although this dual specificity has not been previously recognized, there are indications that the procarboxypeptidase described here is identical with the procarboxypeptidase B partially purified by Folk and Gladner (1958). For this reason, this proenzyme will be referred to as bovine pancreatic procarboxypeptidase B.

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[1] The following abbreviations are used here: DEAE = diethylaminoethyl-, Tris = tris(hydroxymethyl)aminomethane, DFP = diisopropylphosphofluoridate, HPLA = hippuryl-*d,l*-phenyllactic acid, BGA = benzoylglycyl-L-arginine, CGP = carbobenzoxyglycyl-L-phenylalanine.

Materials and Methods

Acetone powders, prepared from fresh bovine pancreas glands by the method described by Stein (1954) and by Keller *et al.* (1956), were obtained from Worthington Biochemical Corporation.

DEAE-cellulose[1] with a capacity of 0.9 meq per gram of resin was a reagent grade product

of the Brown Company. The adsorbant was washed and equilibrated as follows: 150 g DEAE-cellulose was suspended in 4 liters of 0.5 M NaOH–0.25 M NaCl and stirred at room temperature for 1 hour. The suspension was then centrifuged at 2000 rpm (International Centrifuge PR 2, No. 276 A rotor) and the resin resuspended in 6 liters of distilled water. After the suspension was allowed to settle for half an hour, the fines were decanted and the resin filtered through a Buechner funnel. This procedure was repeated once, followed by six additional washings, each with 4 liters of distilled water. Washed DEAE-cellulose was resuspended in 3.5 liters of 0.01 M Tris-HCl, *p*H 7.0, and titrated to *p*H 7.0 by the dropwise addition of concentrated HCl. The resin was filtered again and finally resuspended in 3.5 liters of 0.01 M Tris-HCl, *p*H 7.0. The *p*H of the slurry was readjusted to 7.0 if necessary. Equilibrated DEAE-cellulose was suspended in 0.01 M Tris-HCl, *p*H 7.0, and stored at 4° until use.

DEAE-Sephadex A-50 medium, with a capacity of 3.9 meq per gram, was purchased from Pharmacia, Uppsala, Sweden, and was washed and equilibrated as follows: 10 g of resin was suspended in 500 ml of distilled water and stirred occasionally. After the suspension was allowed to settle for 30 minutes the fines were decanted, and the resin was filtered through a Buechner funnel, suspended in 500 ml of 0.5 N HCl, and stirred for 15 minutes at room temperature. DEAE-Sephadex was then filtered and was washed by suspending the adsorbant in 500 ml of distilled water and filtering it again. The water washing was repeated 6–8 times. The adsorbant was resuspended in 500 ml of 0.5 N NaOH, stirred for another 15 minutes at room temperature, and then filtered and washed 6–8 times with water. Washed DEAE-Sephadex was finally suspended in 500 ml of 0.01 M Tris-HCl, *p*H 8.0, and titrated to *p*H 8.0 with concentrated HCl. The resin was filtered again and resuspended in 500 ml of 0.01 M Tris-HCl, *p*H 8.0. The *p*H of the slurry was readjusted to 8.0 if necessary. Equilibrated DEAE-Sephadex, suspended in 0.01 M Tris-HCl, *p*H 8.0, was stored at 4° until use.

Diisopropylphosphofluoridate (DFP) was purchased from Aldrich Chemical Company, Inc., and was used without dilution.

Trypsin, once crystallized and containing 50% $MgSO_4$, and lyophilized crystalline trypsin (salt free) were obtained from Worthington Biochemical Corporation.

Carboxypeptidase A was prepared in these laboratories (Cox *et al.*, 1962b).

Benzoylglycyl-L-arginine (BGA) was obtained from Dr. Roger Roeske of the Lilly Research Laboratories.

Carbobenzoxyglycyl-L-phenylalanine (CGP) was purchased from Mann Research Laboratories.

Hippuryl-dl-phenyllactic acid (HPLA) was prepared by Mr. W. O. McClure in these laboratories.

All salts used were reagent grade.

*p*H was measured at room temperature with a Radiometer type PHM 22p *p*H meter with a glass electrode.

Protein concentrations were calculated from the optical density at 280 mμ. The determination of $E^{1\%}_{280}$ for procarboxypeptidase B (16) and for carboxypeptidase B (21), is described in the accompanying paper (Cox *et al.*, 1962c).

Protein concentrations in the chromatographic fractions were determined by the method of Lowry *et al.* (1951), with use of the automatic sampler and recorder of a Technicon Autoanalyzer. Measurements were done at 660 mμ.

Enzyme Assay.—Carboxypeptidase B activity was routinely determined with HPLA as substrate. A 0.01 M solution of *dl*-HPLA in 0.045 M NaCl–0.005 M Na-barbital buffer at *p*H 7.5 was used. Since only the *l*-isomer of HPLA is hydrolyzed, the actual substrate concentration was only 0.005 M. Ten μl of a carboxypeptidase B solution containing 0.2 to 0.8 mg/ml was added to 3.0 ml of substrate solution and hydrolysis of the substrate was measured by continuous titration in a *p*H-stat at 25°. Titration was carried out with 0.1 M NaOH to an end-point of *p*H 7.5.

Activity of carboxypeptidase B against basic substrates was estimated with an 0.001 M solution of BGA in 0.045 M NaCl–0.005 M Na-barbital buffer *p*H 7.5. Ten μl of a carboxypeptidase B solution containing 0.1 to 0.3 mg/ml was added to 3.0 ml of the substrate solution at 25°, and 0.2-ml aliquots were removed at 2-minute intervals and pipetted into 2.0 ml of ninhydrin reagent (Matheson *et al.*, 1961), mixed, and heated for 20 minutes in a boiling water bath. After cooling and dilution with 10 ml of 50% ethanol, the ninhydrin color was read at 570 mμ. Activity against CGP was determined by a similar method. The substrate solution was 0.025 M CGP in 0.1 M NaCl–0.02 M Na-barbital buffer, *p*H 7.5. Ten to 25 μl of a carboxypeptidase B solution containing 0.2 to 1.0 mg/ml was added to 1.2 ml of the substrate solution at 25°, and 0.2-ml aliquots were taken at 2-minute intervals for assay of the ninhydrin color.

Results

Isolation of Procarboxypeptidase B

Extraction.—One hundred fifty g of acetone powder of bovine pancreas glands was suspended in 1500 ml of cold distilled water containing 0.5 ml of *n*-octanol (added to suppress foaming) and 0.3 ml of DFP. All operations were carried out at 4° unless otherwise stated. The *p*H of the suspension was adjusted to 6.5, the mixture stirred mechanically, and the *p*H of the slurry readjusted to 6.5 every hour. After 2 hours, another 0.3 ml of DFP was added. The extraction was continued for a total of 4 hours and the suspension was then centrifuged for 30 minutes at 18,000

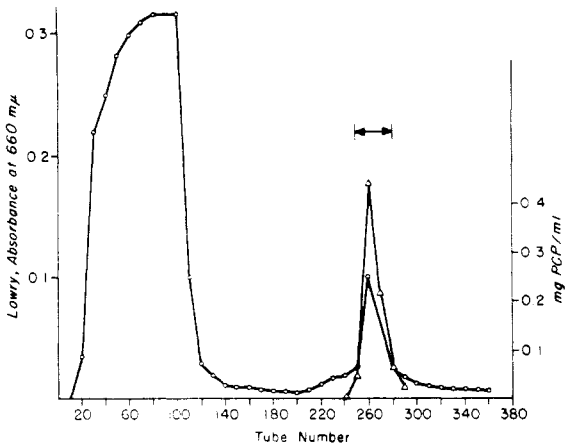

FIG. 1.—Chromatography of an aqueous extract of acetone powder of bovine pancreas on DEAE-cellulose in 0.01 M Tris-HCl–0.02 M NaCl, *p*H 7.0. Triangles represent activity, measured after tryptic activation with HPLA as substrate. The values for mg carboxypeptidase B per ml obtained by this assay were multiplied by the factor 1.69 to give mg procarboxypeptidase B per ml. The fractions within the horizontal arrow were combined. At fraction 360 a linear gradient from 0.02 M NaCl to 0.4 M NaCl was applied for recovery for procarboxypeptidase A.

rpm in a Spinco Model L ultracentrifuge, No. 21 rotor. The clear supernatant was adjusted to *p*H 7.0 by dropwise addition of 5 N NaOH and dialyzed overnight against 40 liters of distilled water.

Chromatography on DEAE-Cellulose (Fig. 1).—Equilibrated DEAE-cellulose, suspended in 0.01 M Tris-HCl, *p*H 7.0, was evacuated with the water aspirator to remove dissolved air and was then poured at room temperature into a column (4.5 cm, i.d.). The resin was allowed to settle under gravity to a height of about 70 cm. After pressure was applied from a regulated air line (5 p.s.i.) for further packing, a column with a height of about 54 cm resulted. The column was then placed in the cold room and washed overnight with 0.01 M Tris, *p*H 7.0.

To the dialyzed extract was added 10 ml of 1 M Tris-HCl, *p*H 7.0, and 1.17 g of NaCl per liter of extract, resulting in a final concentration of 0.01 M Tris and 0.02 M NaCl. The *p*H was adjusted to 7.0 and the extract was then pumped onto the column at a constant flow rate of 160 ml per hour. Fractions of 20 ml volume were collected. Seven liters of 0.01 M Tris–0.02 M NaCl, *p*H 7.0, were then pumped through the column at the same flow rate and the protein concentration of every fifth fraction of the effluent was determined by the Lowry method. Assay for procarboxypeptidase B was performed after activation of 0.5-ml aliquots of the fractions with 10 μl of a trypsin solution (8 mg of a trypsin preparation containing 50% $MgSO_4$ per ml of 0.001 M HCl) at 37° for 1½ hours. HPLA or BGA was used as substrate. Fractions containing procarboxypeptidase B were combined, omitting those fractions which contained less than 10% of the activity present at the maximum of the peak, as indicated in Figure 1. In order to reduce the volume of the solution, the protein was precipitated by addition of solid ammonium sulfate (423 g per liter of effluent). During this operation, the *p*H was maintained at 7.0 by adding a few drops of 5 N NaOH. After the suspension was stirred for 1 hour the precipitate was collected by centrifugation at 18,000 rpm for 30 minutes (Spinco Model L ultracentrifuge, No. 21 rotor). The supernatant was discarded and the residue was dissolved in approximately 10 ml of 0.01 M Tris-HCl, *p*H 8.0, and dialyzed against several changes of the same buffer.

Rechromatography of Partially Purified Procarboxypeptidase B on DEAE-Sephadex (Fig. 2).—A suspension of equilibrated DEAE-Sephadex in 0.01 M Tris-HCl, *p*H 8.0, was deaerated thoroughly and poured at room temperature into a column of 2 cm i.d. to a height of 8 cm. The column was then placed in the cold room and washed for several hours with 0.01 M Tris-HCl, *p*H 8.0. The dialyzed solution of partially purified procarboxypeptidase B was centrifuged to remove some insoluble material and the supernatant was then applied to the resin. A linear gradient ranging from 0.01 M Tris-HCl, *p*H 8.0, to 0.01 M Tris-HCl, 0.2 M NaCl, *p*H 8.0, was applied for elution of the protein. The flow rate of the column was adjusted to 18 to 20 ml per hour, and 6-ml fractions were collected. The protein concentration of every second effluent fraction was determined by the Lowry method. The fractions of the main peak were combined, omitting those fractions which contained less than 15–20% of the protein present at the maximum of the peak, as shown in Figure 2. The solution was again concentrated by precipitation of the protein with ammonium sulfate (42.3 g solid ammonium sulfate per 100 ml of the effluent). During the addition of the salt, the *p*H was allowed to drop from 8.0

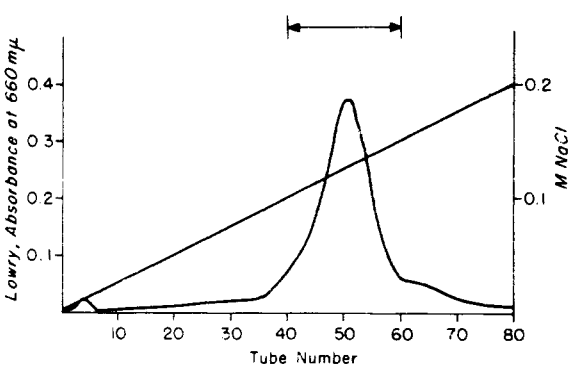

FIG. 2.—Rechromatography of partially purified procarboxypeptidase B on DEAE-Sephadex A-50, medium, in 0.01 M Tris-HCl, *p*H 8.0. A linear gradient from 0 to 0.2 M NaCl was applied for elution of the protein. The fractions within the horizontal arrow were combined.

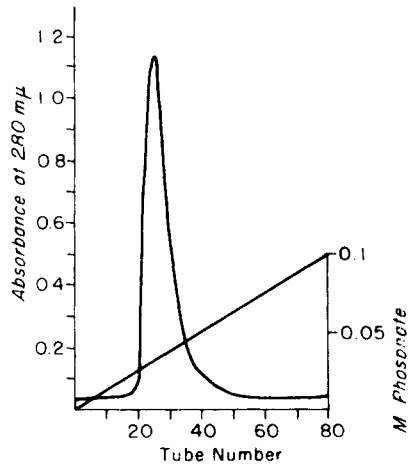

FIG. 3.—Chromatography of purified procarboxypeptidase B on DEAE-cellulose in phosphate buffer at *p*H 8.0. A linear gradient from 0.001 to 0.1 M phosphate was applied for elution of the protein.

to approximately 7.0. The precipitate collected by centrifugation at 15,000 rpm (International Centrifuge HR-1, No. 856 rotor) for 20 minutes was dissolved in approximately 3 ml of 0.001 M potassium phosphate buffer, *p*H 8.0, and dialyzed versus the same buffer.

Properties of Purified Procarboxypeptidase B

Procarboxypeptidase B prepared as described had no detectable intrinsic carboxypeptidase B activity and was stable at 4° for 2 to 4 weeks, after which time slow activation occurred. Lyophilization resulted in considerable inactivation. The protein was soluble in water or buffers above *p*H 6.2 or below *p*H 5.8. Solutions of the protein gave a single, homogeneous peak when subjected to chromatography on DEAE-cellulose in phosphate buffer, *p*H 8.0 (see Fig. 3). The protein also appeared homogeneous during sedimentation in the ultracentrifuge (Spinco Model E) and during moving boundary electrophoresis (Spinco Model H apparatus) as shown in Figure 4. The sedimentation coefficient, $s_{20,w}$, was 4.0 Svedberg units and was independent of the protein concentration in the range from 3 to 12 mg protein/ml.

The molecular weight, determined by short column sedimentation equilibrium, was 57,400 ± 1000, assuming a partial specific volume of 0.73 cm^3/g.

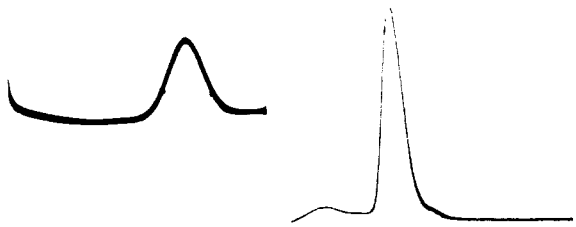

FIG. 4.—*Left*, sedimentation of purified procarboxypeptidase B in 0.1 M potassium phosphate, *p*H 8.0. The protein concentration was 12 mg per ml. The pattern shown was obtained after 70 minutes at 59,780 rpm (bar angle 70°). Sedimentation is from right to left. *Right*, electrophoresis of purified procarboxypeptidase B in Tris-HCl, *p*H 8.0, ionic strength 0.1. Pattern obtained from the descending limb after 305 minutes of electrophoresis at a field strength of 5.02 volts per cm.

Activation of Procarboxypeptidase B

Activation of procarboxypeptidase B was effected by trypsin. As shown in Figure 5, activity toward the substrates BGA and HPLA appeared in a parallel fashion. A molar ratio of trypsin to procarboxypeptidase of 1:1000 was sufficient to catalyze the appearance of 60–70% of maximum activity within 3 hours at 25°. Activation under these conditions of very low trypsin concentration was accelerated by the addition of Ca^{++}, probably because of the stabilization of trypsin by the metal. No effect of Ca^{++} could be observed at higher trypsin concentrations.

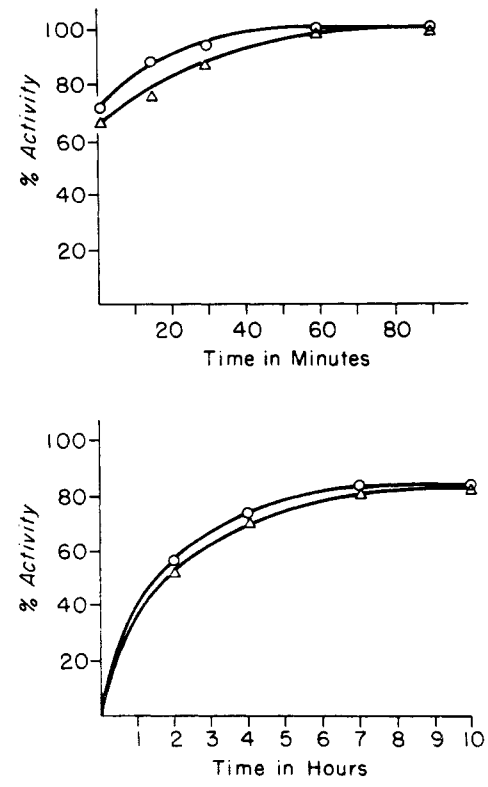

FIG. 5.—Activation of procarboxypeptidase B with trypsin at 25°, *p*H 8.0. Circles represent activities obtained with HPLA as substrate, triangles with BGA as substrate. *Lower*, activation at a molar ratio of trypsin-procarboxypeptidase of 1:1000 in presence of 0.02 M $CaCl_2$ (see text). *Upper*, activation at a molar ratio of trypsin-procarboxypeptidase of 1:10 without $CaCl_2$. The first points were obtained after 1 minute.

On activation with high trypsin concentrations (molar ratio of trypsin to procarboxypeptidase, 1:10), 60–70% of maximum activity occurred within 1 minute and full activity was obtained after approximately 2–3 hours at 25°. Results of sedimentation studies during different stages of activation are shown in Table I. No change

TABLE I

EFFECT OF ACTIVATION OF PROCARBOXYPEPTIDASE B ON SEDIMENTATION COEFFICIENT[a]

	% Activity[b]	$s_{20,w}$
Procarboxypeptidase B	0	4.0 *S*
Procarboxypeptidase B activated with 1:1000 trypsin for 2 hours at 25°, *p*H 8.0	65	4.0 *S*
Procarboxypeptidase B activated with 1:10 trypsin for 1 min. at 25°, *p*H 8.0. Activation stopped by addition of soybean trypsin inhibitor	61	4.0 *S*
Procarboxypeptidase B activated with 1:10 trypsin for 2 hours at 25°, *p*H 8.0	100	3.6 *S*
Carboxypeptidase B	100	3.5 *S*

[a] Procarboxypeptidase B or carboxypeptidase B concentrations were 12 mg/ml in all experiments. [b] Activities were measured prior to sedimentation analysis.

in sedimentation coefficient was observed when procarboxypeptidase B was activated to 60–70% of maximal activity either by incubation at a trypsin-procarboxypeptidase B ratio of 1:1000 for 2 hours at 25°, or by incubation at a trypsin-procarboxypeptidase ratio of 1:10 at 25° for 1 minute. Activation under conditions which gave rise to full activity (trypsin-procarboxypeptidase, 1:10, 2 hours at 25°) was accompanied by a decrease of the sedimentation coefficient from 4.0 to 3.6 Svedberg units.

Crystallization of Carboxypeptidase B

Carboxypeptidase B was crystallized after tryptic activation of the purified proenzyme. A solution of procarboxypeptidase B (approximately 10 mg/ml) in 0.1 M potassium phosphate, *p*H 8.0, was incubated with trypsin at 37° for 5 hours at a molar ratio of approximately 1 trypsin to 10 procarboxypeptidase and the activation mixture was then dialyzed against 0.01 M Tris-HCl, *p*H 8.0. After a few hours, crystallization started. Dialysis was continued for about 48 hours with several changes of the buffer solution. The crystals (Fig. 6) were centrifuged off and dissolved in a small volume of 1 M NaCl, and some insoluble material was removed by centrifugation. For recrystallization, the solution was then dialyzed a second time against 0.01 M Tris-HCl, *p*H 8.0. When crystallization was repeated a third time, no change in the specific activity of

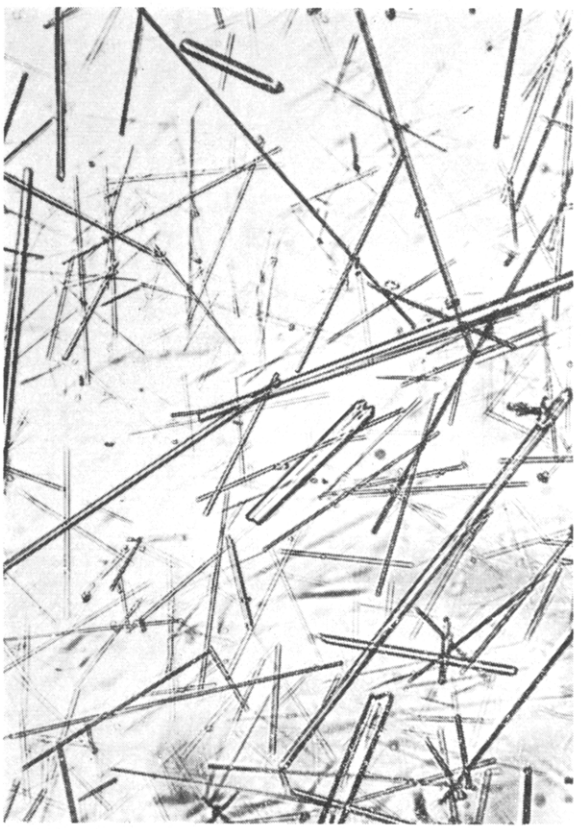

FIG. 6.—Crystalline habit of bovine carboxypeptidase B.

the enzyme or in its amino acid composition was observed, indicating that the enzyme was pure after two crystallizations. The specific activity was identical with that predicted from the specific activity of the proenzyme and from the ratio of the molecular weights of the enzyme and proenzyme.

Carboxypeptidase B thus prepared could be stored indefinitely as a suspension in 0.01 M Tris-HCl, *p*H 8.0, at 4°. The protein was only slightly soluble in water but dissolved readily in 1 M NaCl. The sedimentation pattern obtained with twice-crystallized carboxypeptidase B was consistent with homogeneity (Fig. 7). The sedimentation coefficient increased with increasing protein concentration within the range from 3 to 12 mg protein per ml. Linear extrapolation to zero protein concentration yielded an $s_{20,w}$ of 3.1 Svedberg units.

The molecular weight, determined by short column sedimentation equilibrium, was 34,000, assuming a partial specific volume of 0.73 cm^3/g.

Substrate Specificity of Crystalline Carboxypeptidase B

Carboxypeptidase B obtained as just described was found to hydrolyze substrates containing either basic or aromatic side-chains. The following experiments were performed mainly to com-

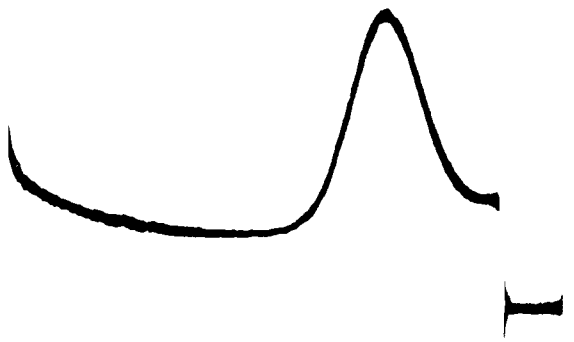

FIG. 7.--Sedimentation of carboxypeptidase B in 1 M NaCl-0.05 M potassium phosphate, *p*H 8.0. The protein concentration was 13 mg per ml. The pattern shown was obtained after 66 minutes at 59,780 rpm (bar angle 70°). Sedimentation is from right to left.

pare the activities of carboxypeptidase B and carboxypeptidase A toward BGA, HPLA, and CGP. Assay conditions were therefore those normally used for the determination of carboxypeptidase A activity. The *p*H of the substrate solutions was 7.5, which is within the rather broad *p*H range where optimal activity of carboxypeptidase B is observed (Folk and Gladner, 1958).

Hydrolysis of BGA.—Units of activity toward BGA are defined as per cent hydrolysis per minute at 0.001 M substrate concentration (Folk *et al.*, 1960). The specific activity, expressed as units per mg of enzyme per ml, was found to be 10,500 ± 500, as compared to 18,000 previously reported for porcine carboxypeptidase B (Folk *et al.*, 1960). The same value was obtained with the ninhydrin method described in this paper and with the spectrophotometric assay described by Folk *et al.* (1960). No hydrolysis of BGA by carboxypeptidase A was observed under conditions where a specific activity of carboxypeptidase A toward the basic substrate 0.3% of that of carboxypeptidase B would have been detected.

Hydrolysis of HPLA.—The zero-order rate constant for the hydrolysis of HPLA was found to be 0.22 ± 0.01 mmoles of substrate hydrolyzed per minute per mg of enzyme protein. The corresponding values for carboxypeptidase A are 0.21 to 0.22 (Bargetzi *et al.*, 1962), indicating that the specific activities of both enzymes toward HPLA are essentially the same.

Hydrolysis of CGP.—The proteolytic coefficient calculated for carboxypeptidase B, assuming first-order kinetics, is 0.64 min^{-1} per mg of protein per ml. The value obtained with carboxypeptidase A was 3.3 (Bargetzi *et al.*, 1962). Carboxypeptidase B is therefore only about 20% as active toward CGP as is carboxypeptidase A.

Experiments were designed to exclude the possibility that the activities of carboxypeptidase B against HPLA and CGP were due to contamination by carboxypeptidase A. The lysine analogue, ε-amino-caproic acid, is a potent inhibitor of carboxypeptidase B activity against its basic substrates (Folk, 1956). As shown in Table II, the *basic* inhibitor also suppressed the hydrolysis of the *aromatic* substrate HPLA by bovine carboxypeptidase B. In contrast, the activity of carboxypeptidase A toward HPLA was completely unaffected by ε-amino-caproic acid, even as an inhibitor to substrate ratio of 10:1, which is 20 times the ratio that produces 80% inhibition of carboxypeptidase B.

TABLE II

INHIBITION OF CARBOXYPEPTIDASE B ACTIVITY TOWARD HPLA BY ε-AMINO-CAPROIC ACID

The substrate solution was that described in Methods. The controls contained appropriate concentrations of NaCl to give equal ionic strengths in experiments with and without inhibitor.

Substrate Concentration (10^{-3} M)	Inhibitor Concentration (10^{-1} M)	Inhibitor Concentration / Substrate Concentration	% Inhibition
5.0	3.3	1/15	35
5.0	10.0	1/5	63
5.0	25.0	1/2	81

Carboxypeptidase A activity against HPLA is inhibited by the phenylalanine analogue β-phenylpropionic acid (Elkins-Kaufmann and Neurath, 1949). As shown in Table III, the activities of carboxypeptidase A and carboxypeptidase B

TABLE III

INHIBITION OF THE ACTIVITIES OF CARBOXYPEPTIDASE A AND CARBOXYPEPTIDASE B TOWARD HPLA BY β-PHENYLPROPIONIC ACID

The buffer was that described in Methods. The controls contained appropriate concentrations of NaCl to equal ionic strengths in experiments with and without inhibitor.

Substrate Concentration (10^{-3} M)	Inhibitor Concentration (10^{-3} M)	Inhibitor Concentration / Substrate Concentration	% Inhibition: Carboxypeptidase A	% Inhibition: Carboxypeptidase B
5.0	2.5	1/2	18	15
4.8	4.8	1	43	51
4.0	20.0	5	92	90

TABLE IV

INHIBITION OF CARBOXYPEPTIDASE B ACTIVITY TOWARD BGA BY β-PHENYLPROPIONIC ACID

The substrate was dissolved in 0.045 M NaCl–0.005 M Na-barbital buffer, *p*H 7.5, as described in Methods. The same buffer was used for dilution in experiments with lower substrate concentration. An additional 0.05 M NaCl was added to the controls to bring the ionic strength of the controls up to that of the samples containing the inhibitor.

Substrate Concentration (10^{-3} M)	Inhibitor Concentration	Inhibitor Concentration	
	Inhibitor Concentration (10^{-2} M)	Substrate Concentration	% Inhibition
5.0	5.0	10	31
2.5	5.0	20	55
1.0	5.0	50	77

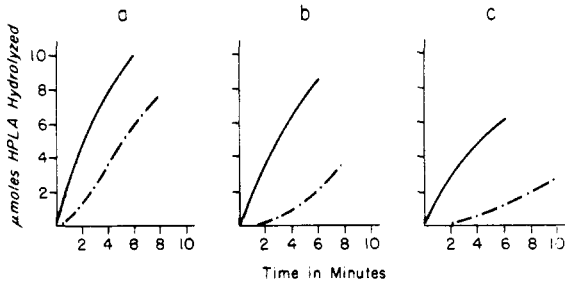

FIG. 8.—Hydrolysis of HPLA by carboxypeptidase B in the presence of BGA. *a*, HPLA = 4.5×10^{-3} M, BGA = 0.9×10^{-3} M. *b*, HPLA = 3.75×10^{-3} M, BGA = 2.5×10^{-3} M. *c*, HPLA = 3.3×10^{-3} M, BGA = 3.3×10^{-3} M. Solid lines represent controls without BGA.

toward HPLA were suppressed in a parallel fashion by this inhibitor. Thus, the two enzymes not only have the same specific activity toward HPLA but are also inhibited to the same extent by β-phenylpropionic acid. Additional experiments, summarized in Table IV, show that the *aromatic* inhibitor also suppressed carboxypeptidase B activity toward the *basic* substrate BGA.

Another experiment relevent to the same question is shown in Figure 8. Here the hydrolysis of HPLA by carboxypeptidase B was measured by continuous titration in the presence and absence of BGA. The hydrolysis of the ester was strongly inhibited in the presence of BGA as compared to controls without BGA, and the inhibition was relieved as the basic substrate was hydrolyzed.

Companion experiments with carboxypeptidase A revealed no inhibition of hydrolysis of HPLA by BGA. In the reverse experiment with carboxypeptidase B, no inhibition by HPLA of the hydrolysis of BGA could be demonstrated at an HPLA-BGA ratio of 5:1. Higher ratios of HPLA to BGA were not examined.

Inhibition by 1,10-Phenanthroline

Bovine carboxypeptidase A (Vallee and Neurath, 1955) and porcine carboxypeptidase B (Folk *et al.*, 1960) are zinc metalloenzymes and are inhibited by metal chelating agents such as 1,10-phenanthroline. When crystalline bovine carboxypeptidase B was preincubated with 10^{-3} M 1,10-phenanthroline for 1 hour at 0° and its activity was subsequently measured in substrate solutions containing the same inhibitor concentration, 90% inhibition was observed. Similar observations have been made previously on this enzyme by Haynes (1961). Activity could be partially recovered by dilution of the inhibited enzyme with substrate solutions containing no inhibitor.

DISCUSSION

A procedure for the purification (20-fold) of procarboxypeptidase B from acetone powders of beef pancreas glands has been described previously (Folk and Gladner, 1958). The procedure included isoelectric precipitation, treatment with an ion exchange resin, extraction of inactive material with barium hydroxide at *p*H 6, and finally extraction of procarboxypeptidase with sodium chloride. The resulting preparation was found to be rather unstable, activity being rapidly lost on storage at −10°, or on dialysis. Further purification of the preparation described by these authors was achieved by Pechère *et al.* (1958) by use of chromatography on DEAE-cellulose. More recently, Haynes (1961) has also succeeded in isolating bovine procarboxypeptidase B in relatively pure form by chromatography on DEAE-cellulose, using an ammonium sulfate precipitate of aqueous extracts of acetone powder as starting material. The preparation described by Haynes was found to be monodisperse in the ultracentrifuge and on activation gave rise to carboxypeptidase B of high specific activity.

The purification of bovine procarboxypeptidase B described in this report involves only chromatographic procedures and avoids conditions of low *p*H, since these seem to cause instability in the protein. When chromatography of the crude acetone powder extract was carried out under conditions described for the preparation of procarboxypeptidase A (no prior dialysis of the extract, chromatography on DEAE-cellulose with a phosphate gradient at *p*H 8.0), procarboxypeptidase B was found to emerge with the "breakthrough" peak together with trypsinogen and chymotrypsinogen (Yamasaki *et al.*, 1962). Under these conditions, the resolution obviously is poor and the zymogen becomes easily activated by small amounts of active trypsin present in this fraction. It was necessary, therefore, to find conditions for the greater retention of the zymogen by the adsorbant in order to separate this proenzyme from the proteins of the "breakthrough" peak. The chromatographic procedure described in this report fulfills this requirement.

As with all procedures for the isolation of pancreatic zymogens, preparations of acetone powder of high quality, containing no detectable active trypsin, are prerequisite for the success of the present method.

When, after emergence of procarboxypeptidase B from the column, a salt gradient was applied in order to recover procarboxypeptidase A, another fraction was observed to emerge which, after activation, also displayed activity against the basic substrate BGA. This fraction appeared just ahead of chymotrypsinogen B and contained approximately one third to one half of the potential carboxypeptidase B activity found in the main peak. It is likely that this fraction was identical with the main fraction, despite the fact that it displayed different chromatographic behavior. Thus the ratios of activities against HPLA and BGA were the same for both fractions. When the two fractions were rechromatographed separately on DEAE-Sephadex, activity appeared at the same salt concentration, but fraction II was still inhomogeneous. The proportion of the activity obtained in the two fractions varied as a function of the column load in the first chromatography, fraction II remaining constant while fraction I increased as the column load was increased. These results are consistent with the interpretation that the two peaks represent an artifact of chromatography rather than two different species of procarboxypeptidase B. Attempts to eliminate entirely the chromatographic artifact, or to purify the procarboxypeptidase B eluted in fraction II, have thus far been unsuccessful. The first chromatography, therefore, involves the loss of about 30% of the procarboxypeptidase B activity. The entire procedure does, however, lead to the recovery of pure enzyme accounting for 50% of the total potential activity against BGA detectable in the crude extract. In routine operations, approximately 100–150 mg of procarboxypeptidase B has been obtained in pure form from 150 g of acetone powder.

Purified procarboxypeptidase B appears to be a homogeneous protein as judged by sedimentation analysis, electrophoresis, chromatography, and potential enzymatic activity. Additional chromatography of the purified protein on DEAE-cellulose had no effect on these parameters.

The successful isolation of procarboxypeptidase B in pure form made it possible for the first time to study in detail the nature of the activation process. Tryptic activation of the proenzyme under mild conditions (trypsin-zymogen mole ratios of 1:1000) does not give rise to full activity, but a plateau is reached lying at from 60–80% of the maximal activity. Activation with larger amounts of trypsin (trypsin-zymogen, 1:10) is almost instantaneous up to 60–70% of maximal activity and occurs much more slowly thereafter. Sedimentation analyses have shown a similarity between the products obtained on activation up to 60–70% of maximum activity by either procedure. The sedimentation coefficient remained unchanged during activation under such mild conditions and was the same as that of pure procarboxypeptidase B. However, if the proenzyme was fully activated by prolonged exposure to a trypsin-procarboxypeptidase ratio of 1:10, the sedimentation coefficient decreased to a value similar to that observed with crystalline carboxypeptidase B. These findings suggest that activation occurs in two stages. The first step is completed rapidly and leads to a protein which is approximately 60–70% as active as carboxypeptidase B and which has the same sedimentation coefficient as the proenzyme. It is likely that, during the first step, only the most susceptible bond or bonds of the zymogen molecule are split by trypsin, leading to rearrangement of the tertiary structure and to activation of the zymogen. Since this event has no detectable effect on the sedimentation coefficient, one may suppose that this step does not involve any significant fragmentation of the molecule. Only after treatment with trypsin for longer periods is part of the proenzyme molecule removed, thus leading to the formation of carboxypeptidase B which now possesses full activity. Results of end-group and amino acid analyses reported in the accompanying paper are consistent with this interpretation (Cox *et al.*, 1962c).

Earlier studies in this laboratory have shown that bovine procarboxypeptidase A is a zymogen both for carboxypeptidase A and for an endopeptidase which hydrolyzes acetyl-L-tyrosine ethyl ester. The endopeptidase as well as trypsin was found to be essential for the conversion of the zymogen to carboxypeptidase A (Keller *et al.*, 1958a). Attempts to detect an analogous endopeptidase activity during the activation of bovine carboxypeptidase B have been unsuccessful. No hydrolysis of acetyl-L-tyrosine ethyl ester was observed under any conditions of activation, nor did the rate of hydrolysis of benzoyl-L-arginine ethyl ester exceed that expected of the trypsin added, suggesting that the conversion of this zymogen is mediated only by trypsin.

When pure procarboxypeptidase is fully activated by trypsin and the activation mixture is dialyzed, crystallization of carboxypeptidase B ensues. Since the corresponding enzyme from porcine pancreas (Waldschmidt-Leitz *et al.*, 1931; Weil *et al.*, 1959; Folk *et al.*, 1960) has thus far not been crystallized, this is the first report of the crystallization of an enzyme having the specificity of carboxypeptidase B.

The term "carboxypeptidase B" has been applied to those exopeptidases which hydrolyze substrates containing C-terminal lysine or arginine (Folk, 1956). Such substrates are not hydrolyzed by carboxypeptidase A (Neurath, 1960). It has been suggested that the specificities of the two carboxypeptidases are mutually exclusive and that carboxypeptidase B hydrolyzes only basic substrates (Folk and Gladner, 1961; Weil *et al.*,

1959; Desnuelle and Rovery, 1961). Crystalline carboxypeptidase B from bovine pancreas, however, is as active against the aromatic ester HPLA as is carboxypeptidase A; in addition, it hydrolyzes the peptide substrate CGP one fifth as rapidly as the A enzyme. These activities are not due to contamination of the preparation with carboxypeptidase A, since the B and A enzymes are equally active against HPLA. Moreover, the HPLA-splitting activity of carboxypeptidase B is strongly inhibited by ϵ-amino-caproic acid or by BGA, whereas the corresponding activity of carboxypeptidase A is entirely unaffected by either compound. The fact that the activity of carboxypeptidase B against BGA is inhibited by β-phenylpropionate is a further indication that the affinities for both basic and aromatic compounds reside in the same enzyme. The relative concentrations of substrate and inhibitor required to demonstrate inhibition in the BGA-β-phenylpropionate and HPLA-ϵ-amino caproate systems suggest that the basic compounds are bound to the enzyme considerably more strongly than the aromatic substrate or inhibitors. This conclusion is supported by the inhibition of HPLA hydrolysis by BGA and would account for the failure to demonstrate inhibition of BGA hydrolysis by HPLA at an HPLA-BGA ratio as high as 5:1. The evidence at hand thus indicates that the activity of bovine carboxypeptidase B toward aromatic substrates is an intrinsic property of this enzyme and that the specificities of the carboxypeptidase A and B are not mutually exclusive. Whereas carboxypeptidase A does not hydrolyze basic substrates to any detectable degree, carboxypeptidase B, although most active toward basic substrates, in addition hydrolyzes substrates for carboxypeptidase A at a considerable rate. It is not possible to decide from the present experimental data whether these two specificities are mediated by the same active center or whether they are due to different but overlapping centers. It is evident, however, that both activities are abolished by chelating agents such as 1,10-phenanthroline (Haynes, 1961) and that a metal (zinc) is an essential and functional component of the active site (Cox *et al.*, 1962c).

It is possible that there might be present in bovine pancreas another carboxypeptidase with an absolute requirement for basic substrates. Such a possibility is rendered doubtful by the fact that the yield of the purified proenzyme described herein accounts fairly well for the potential activity against BGA in the actone powder extract from which it is isolated. Moreover, no other protein with activity against the basic substrate has been removed in the course of the purification. Most important, the published data do not require that the bovine carboxypeptidase B hydrolyze only basic substrates; the bovine enzyme has not previously been purified to a degree sufficient that a critical test for the presence of intrinsic carboxypeptidase A activity could be made (Gladner and Folk, 1958). The information available indicates that the specificity of the bovine carboxypeptidase B differs from that reported for the analogous porcine enzyme. Although the specificity of the porcine enzyme is apparently absolute, that of the bovine enzyme is not.

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Bovine Pancreatic Procarboxypeptidase B. II. Mechanism of Activation*

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The present report extends a preceding investigation of the isolation, characterization, and activation of bovine pancreatic procarboxypeptidase B (Wintersberger *et al.*, 1962). The amino acid composition of the zymogen and of crystalline carboxypeptidase B derived from it is given in detail. The amino terminal residue of carboxypeptidase B has been identified as threonine. The initial stage of activation of procarboxypeptidase B has been examined, and evidence has been obtained that the appearance of activity coincides with the hydrolysis of a single arginyl-threonine linkage in the zymogen. Preliminary data indicate that crystalline bovine carboxypeptidase B, like carboxypeptidase A and porcine carboxypeptidase B, contains approximately one gram atom of zinc per mole (molecular weight 34,000).

In the accompanying paper (Wintersberger *et al.*, 1962), we have reported the isolation of procarboxypeptidase B from acetone powder of bovine pancreas. The protein was found to be homogeneous according to chromatography, sedimentation analysis, electrophoresis, and potential enzymatic activity, and upon activation gave rise to carboxypeptidase B which has been isolated in crystalline form. The active enzyme hydrolyzed substrates containing both aromatic and basic carboxyl-terminal residues, and evidence was presented to suggest that this dual specificity is mediated by the same or overlapping active sites.

The present investigation extends the characterization of bovine pancreatic carboxypeptidase B and its zymogen in terms of amino acid composition and amino-terminal groups. In addition, further details are given of the initial stage of the trypsin-catalyzed reaction which results in the conversion of the zymogen to the active enzyme.

MATERIALS AND METHODS[1]

Bovine procarboxypeptidase B was isolated from acetone powders of beef pancreas and *carboxypeptidase B* was prepared from the purified proenzyme by the methods described in the accompanying paper (Wintersberger *et al.*, 1962).

Carboxypeptidase B activity was determined by the assay for HPLA outlined in the accompanying paper (Wintersberger *et al.*, 1962). Procarboxypeptidase B was estimated from the maximum activity of carboxypeptidase B reached during tryptic activation.

Protein concentrations were measured spectrophotometrically at 280 mμ. Extinction coefficients for procarboxypeptidase B and for carboxypeptidase B were determined by the method of Walsh and Brown (1962). The values obtained for $E^{1\%}_{280}$ were 16 for procarboxypeptidase B and 21 for carboxypeptidase B. These values are considered accurate to within ±0.5.

Amino acid analyses were performed with a Beckman/Spinco Model 120 automatic amino acid analyzer by the method of Spackman *et al.* (1958). Hydrolysis of 5-mg samples was carried out in evacuated, sealed ignition tubes at 105°, in constant-boiling HCl. Each hydrolyzed sample was dried and dissolved in 4.8 ml of 0.2 M citrate buffer. Following the suggestion of Walsh and Brown (1962), 0.2 ml of a standard solution containing thienylalanine and aminoguanidopropionic acid, each at a concentration of 0.01 M, was added to allow correction for unequal recoveries from the long and short columns of the analyzer. Aliquots of 2 ml were taken for analysis on each column. Half-cystine was determined as cysteic acid after oxidation of 5–8 mg samples with performic acid for 11 hours at 0° (Hirs, 1956). Excess performic acid was removed com-

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[1] The following abbreviations are used: FDNP = 1-fluoro-2,4-dinitrobenzene; DNP = dinitrophenyl; Tris = tris(hydroxymethyl)aminomethane; HPLA = hippuryl-*dl*-phenyllactic acid.