Procedures for the Isolation of Crystalline Bovine Pancreatic Carboxypeptidase A.* I. Isolation from Acetone Powders of Pancreas Glands

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A procedure is described for the preparation of crystalline carboxypeptidase A from acetone powders of beef pancreas glands. In this method the inactive precursor, procarboxypeptidase A, together with other pancreatic proteins, is extracted with water and activation is initiated by the addition of trypsin under controlled conditions. Subsequent fractionation includes precipitation with ammonium sulfate and isoelectric precipitation. Crystallization is accomplished by procedures similar to those previously described. The present procedure has the advantage of using a stable starting material and being more nearly reproducible than the methods previously described. The final product, crystalline carboxypeptidase A, resembles the protein isolated by earlier methods in gross chemical composition and specific activity. It possesses a higher solubility, and in contrast to the enzyme prepared by the method of Anson, the present enzyme has been reported to be fully reactivatable upon addition of zinc to the metal-free apoenzyme (B. L. Vallee, T. L. Coombs, and F. L. Hoch (1960), *J. Biol. Chem. 235*, PC 45).

Bovine pancreatic carboxypeptidase A was first isolated in crystalline form by Anson (1935, 1937). The method involved extensive autolysis of the glands, collection of the exudate over a period of several days, and fractional precipitation of the proteins of the fully activated pancreatic exudate. The method is inherently laborious and not always reproducible, since autolysis is ill-controlled and the quantity of exudate varies considerably with the conditions of thawing of the initially frozen glands. Several modifications of the original procedure have been described (Putnam and Neurath, 1946; Neurath *et al.*, 1947; Smith *et al.*, 1949; Neurath, 1955) but all the revised procedures have retained the autolytic activation and its attendant difficulties.

A method of isolation of crystalline carboxypeptidase A has been worked out, different in kind from the preceding ones, relying on stable acetone powder of freshly collected pancreas glands as starting material. In this method the inactive precursor of the enzyme, procarboxypeptidase A, is first extracted together with other pancreatic proteins from the acetone powder, and activation is initiated by the addition of trypsin under controlled conditions. This method of isolation yields crystalline enzyme differing in certain characteristic properties from that obtained by the method of Anson from spontaneously autolyzed tissues. Because the enzyme isolated by the present method has been extensively used in recent years in studies relating structure and function of bovine pancreatic carboxypeptidase A (see Rupley and Neurath, 1960; Vallee *et al.*, 1960a, 1963), this procedure is presented herein in detail. Another method of isolation of carboxypeptidase A from acetone powder yields yet another form of this enzyme and in considerably better yield, and is presented in the companion report (Cox *et al.*, 1964).

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Materials and Methods

Acetone powders were prepared from freshly collected pancreas glands by the method described by Fischer and Stein (1954) and by Keller *et al.* (1956), and stored at −20°. In later stages, some powders were obtained from commercial sources. One g of acetone powder was equivalent to approximately 5 g of ground pancreatic tissue.

Trypsin, twice crystallized and containing 50% $MgSO_4$, was obtained from Worthington Biochemical Corp. A solution free of salt was prepared by dissolving the enzyme in a concentration of 20 mg/ml in 1×10^{-3} M HCl and dialyzing against the same solvent prior to use.

Carbobenzoxyglycyl-L-phenylalanine (CGP)[1] was purchased as the chromatographically pure product from Mann Research Laboratories.

Solutions of barium hydroxide used in the preparative procedures were prepared just prior to use with CO_2-free water.

*p*H was measured at room temperature with a G-202A glass electrode in conjunction with a Radiometer Type pHM 22p *p*H meter. The *p*H of concentrated or salt-free solutions of protein was determined on aliquots which were first diluted with 0.1 M KCl to approximately 0.1% protein.

Protein concentrations were calculated from the absorbancy at 278 mμ, using the value for $E^{1\%}_{1\,cm}$ of 19.4 reported by Vallee *et al.* (1960a). A value for the nitrogen content of 15.4% was used to convert mg protein to mg protein nitrogen (Smith and Stockell, 1954).

Enzyme assays.—The substrate, CGP, was dissolved by the slow addition of 0.1 M NaOH to a weighed amount of the peptide suspended in Veronal buffer (0.021 M sodium Veronal–0.1 M NaCl, *p*H 7.5). The *p*H was maintained at 6.0–6.5 during dissolution of the substrate, then raised to 7.5, and the solution was diluted to the proper volume. Solutions of the enzyme were diluted with the Veronal buffer cited above.

The assay system contained the substrate (0.02 M CGP) in 0.02 M Veronal–0.1 M NaCl, *p*H 7.5, and approximately 2–5 $\times 10^{-4}$ mg enzyme nitrogen per ml. Incubation was carried out at 25°. Aliquots of 0.2 ml were removed at 5- to 10-minute intervals and the

[1] Abbreviations used in this work: CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-phenyllactate.

amount of phenylalanine released from the peptide was measured by its ninhydrin color (Moore and Stein, 1948).

Enzymatic activity was expressed in terms of a proteolytic coefficient, C (Neurath and Schwert, 1950), calculated from the linear portions of first-order reaction plots provided hydrolysis had not exceeded 15%.

Pure carboxypeptidase, assayed under the above conditions, gave C values of approximately 17.[2]

Isolation Procedure

Unless otherwise stated, all operations were carried out at 4°, and the centrifugal forces specified are those which obtain at the average radius of the rotor.

Extraction.—Beef pancreatic acetone powder was extracted using 12 ml of cold distilled water per gram of acetone powder. The suspension was stirred for 12–24 hours and then allowed to settle overnight. A clear extract was obtained using either of the following procedures: (1) centrifugation in a Spinco Model L ultracentrifuge for one hour at 22,000 $\times g$ (15,000 rpm in Rotor No. 21), or, (2) removal of the heavy stroma by centrifugation in the PR-2 International centrifuge at 1000 $\times g$ for 20 minutes, followed by suction filtration through E & D 616 filter paper covered with a moist mat (*ca.* 4 mm) of filter-cell and glass wool.[3] The aqueous extract may be kept at 4° under an atmosphere of toluene or it may be frozen without harmful effects.

Activation.—Extracts prepared as described contain a number of digestive proteins in zymogen form, viz., trypsinogen, the chymotrypsinogens A and B, as well as the procarboxypeptidase A complex. Activation of the complex of procarboxypeptidase A requires trypsin as well as the endopeptidase which is associated with this zymogen (Keller *et al.*, 1958). The trypsin requirement may be met by either *intrinsic* trypsin, i.e., that formed from trypsinogen during aging of the water extracts, or by the addition of crystalline trypsin. The latter procedure is more rapid and has yielded more reproducible results.

In a typical experiment, the extract from 500 g of powder was warmed rapidly to 37° and the *p*H was adjusted to 7.8 with 1 N NaOH. Trypsin (400 mg) was added and the extent of activation of procarboxypeptidase was followed by removing ten microliter aliquots at 30-minute intervals and assaying for activity against CGP. One hour after maximum carboxypeptidase activity was reached (corresponding approximately to 1 mg carboxypeptidase per ml), the whole activation mixture was quickly cooled to 0° in an ice bath.

Precipitation.—The protein was concentrated by precipitation with ammonium sulfate as follows: Solid finely ground ammonium sulfate (391 g) was added slowly with mechanical stirring to each liter of extract, care being taken that each increment of salt was completely dissolved before the next one was added. The *p*H of the mixture was maintained at 7.5 by the addition of 1 N NaOH. The precipitate was allowed to settle overnight, the supernatant fluid was siphoned off, and the precipitate was collected by centrifugation in the Spinco Model L ultracentrifuge at 33,000 $\times g$ (20,000 rpm in the No. 21 rotor) for 1 hour. The protein pellet was dissolved in cold distilled water to a concentration of around 20 mg/ml as estimated from the absorbancy at 278 mμ. If the solution was turbid, it was clarified by centrifugation at 14,000 $\times g$ for 30 minutes (13,000 rpm, Rotor No. 30).

Ammonium sulfate was then removed from the solution by exhaustive dialysis against cold distilled water, i.e., against six changes of a 25-fold excess volume of water. Removal of the salt caused the appearance of a precipitate. When dialysis was completed the entire contents of the dialysis bags were collected for the next step. If necessary, the dialyzed suspension may be frozen at this stage without loss of activity.

Isoelectric Precipitation.—To avoid denaturation of the enzyme from prolonged exposure to the acid conditions with the concomitant loss of metal (Vallee *et al.*, 1960a), this step was done at 0° and as rapidly as possible. Care should be taken to avoid lower *p*H values. The protein concentration of the dialyzed preparation was adjusted to 15 mg/ml with cold distilled water; 0.1 N acetic acid was added dropwise to the suspension with continuous stirring until a *p*H of 5.5 was reached. After 5 minutes of *p*H equilibration the suspension was centrifuged for 30 minutes at 22,000 $\times g$ in the Spinco Model L preparative ultracentrifuge (Rotor No. 21, 15,000 rpm). The next step was started without delay.

*p*H *6 Extraction.*—The precipitate was suspended at 0° in the same volume of cold distilled water as in the previous step. The *p*H was brought to 6.0 by the addition of 0.05 M $Ba(OH)_2$ and maintained there, with mixing, for 2 hours. The precipitate was then collected by centrifugation for 30 minutes at 22,000 $\times g$ in the Spinco Model L ultracentrifuge (15,000 rpm in Rotor No. 21) and resuspended in four-fifths of the previous volume of cold distilled water.

If it is necessary to store the precipitate overnight at this stage, the *p*H should be raised to 7.5 with 0.05 M $Ba(OH)_2$.

Crystallization.—The *p*H[4] of the suspension was raised slowly to 10.0 by the dropwise addition of a cold solution of 0.05 M barium hydroxide and maintained there until most of the precipitate had dissolved, but not longer than 3–4 hours. During the extraction, in order to avoid irreversible denaturation, a *p*H of 10.4 should not be exceeded. The resultant turbid solution was clarified by centrifugation at 33,000 $\times g$ for 30 minutes (22,500 rpm, Spinco Rotor No. 40). The *p*H of the clear supernatant solution was then lowered very slowly by the dropwise addition of 0.1 N acetic acid to the well-stirred solution. As incipient crystallization was *approached* (and this was manifest by a very slight opalescence or slight color change) seeds of crystalline carboxypeptidase were added, mixing was continued for 10–15 minutes, and the solution was then allowed to stand. If crystallization did *not* commence within 2–3 hours, the slow additions of dilute acid and seed crystals were resumed. At the point of incipient crystallization the suspension was again allowed to stand. The *p*H was lowered gradually over a period of several days to 7.5 and the crystals were collected by centrifugation. First crystals were usually tan in color and were 60–70% pure as judged by enzymatic assay.

Recrystallization and Depigmentation.—Further puri-

[2] The value for the proteolytic coefficient depends markedly upon the substrate concentration as well as the ionic strength of the assay mixture (Lumry *et al.*, 1951). Values of 28 and higher have been noted under assay conditions identical to those described herein, except that the concentration of NaCl was 1.0 M rather than 0.1 M (Coleman and Vallee, 1962). The present value is corrected for the revised value of the extinction coefficient of 19.4 (Bargetzi *et al.*, 1963; Simpson *et al.*, 1963) as compared to 23 (Davie and Neurath, 1955).

[3] The authors are grateful to Dr. Joseph E. Coleman for many helpful suggestions concerning the filtration of large volumes of extract.

[4] The *p*H was measured at this step with a Lyphan *p*H paper.

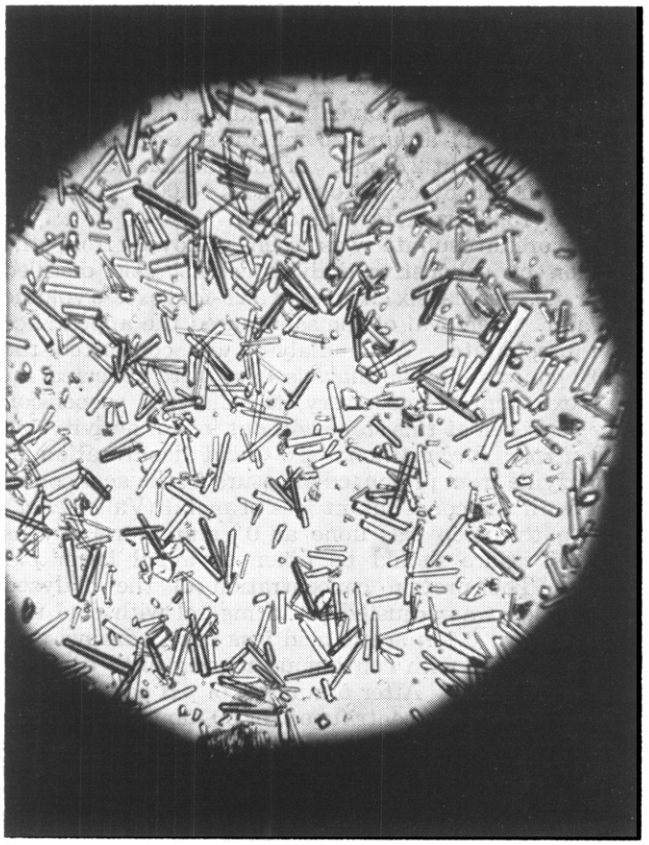

FIG. 1.—The crystal habit of carboxypeptidase A. The crystallization procedure is described in the text.

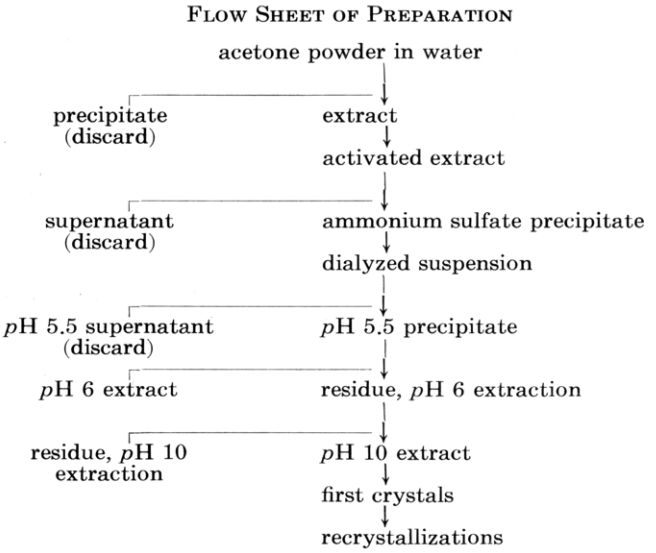

FIG. 2.—Flow sheet showing the major stages in the preparation of carboxypeptidase A from acetone powders of bovine pancreas.

fication and removal of pigment were accomplished by alternative recrystallization procedures.

(1) Crystals were suspended in cold distilled water (around 50 ml/g enzyme). The *p*H of the suspension was raised to 10.0 as described above. After 3–4 hours' extraction, the suspension was centrifuged at 80,000 × *g* (35,000 rpm, No. 40 rotor) in the Spinco Model L ultracentrifuge for 3 hours. The supernatant fluid was decanted and the pigmented pellet was discarded. The *p*H of the supernatant fluid was lowered as described above to yield a crop of recrystallized carboxypeptidase.

(2) Crystals were dissolved in the minimum volume of 1 M NaCl (around 50 ml/g carboxypeptidase). When the crystals had dissolved, the clear solution was dialyzed against 2 liters of each of the following buffers during successive 8-hour periods: (a) 0.5 M LiCl–0.02 M sodium Veronal, *p*H 8.0; (b) 0.2 M LiCl–0.02 M sodium Veronal, *p*H 8.0; (c) 0.1 M LiCl–0.02 M sodium Veronal, *p*H 8.0. Crystals were collected after dialysis vs. the final buffer.

The crystal habits of carboxypeptidase prepared by the present procedure have been studied by Coleman *et al.* (1960). A typical form is depicted in Figure 1. A flow sheet depicting the preparative procedure is shown in Figure 2. A typical protocol is given in Table I.

Alternative Procedure.—In some preparations crystallization did not occur spontaneously when the *p*H was lowered from *p*H 10. In these cases it was found that crystallization could be induced by dialyzing the suspension against four changes of 10 liters of water over a 48-hour period, causing the *p*H to drop to about 7. These crystals were dissolved in 25 ml of 1 M NaCl–0.02 M sodium Veronal, *p*H 8.0, and centrifuged in the No. 40 rotor of the Spinco Model L ultracentrifuge for 16 hours at 40,000 rpm. The brown pellet was discarded. The enzyme was then recrystallized from the pale supernatant solution by dialysis in turn against 2 liters of each of the following solutions: 0.5 M sodium chloride–0.02 M sodium Veronal, *p*H 8.0; 0.2 M sodium chloride–0.02 M sodium Veronal, *p*H 8.0; and 0.1 M sodium chloride–0.02 M sodium Veronal, *p*H 8.0.

Three to five recrystallizations usually yielded material of maximum specific activity.

TABLE I

A TYPICAL PROTOCOL FOR THE PREPARATION OF CARBOXYPEPTIDASE A

Step	Volume (ml)	Total CP[a] (mg)	Total Protein[b] (mg)	*C*[c]	Recovery (%)	Purification
Activated water extract of 500 g of acetone powder	4,680	3,160	199,000	0.28	100	1
Dialyzed ammonium sulfate precipitate	375	2,450	6,400	6.8	81	24
*p*H 5.5 precipitate	330	1,670	3,870	7.6	54	27
*p*H 5.5 supernatant (discard)	365	345	2,200	2.7	—	—
*p*H 6.0 precipitate[d]	262	1,800	3,560	8.4	58	30
*p*H 6.0 supernatant (discard)	318	226	176	2.2	—	—
First crystals	25	1,160	2,030	10	38	36
Fifth crystals	—	—	—	17	—	60

[a] Estimated from enzymatic activity. CP denotes carboxypeptidase. [b] Estimated from optical density measurements at 278 mμ. [c] C denotes proteolytic coefficient. [d] The slight increase in total enzyme and recovery apparent in this step is within the limits of experimental error of activity measurements which served as basis of calculation of yield.

Discussion

The crystalline product obtained by the present procedure has been found in this work and by Rupley and Neurath (1960) to be homogeneous in the ultracentrifuge and during moving-boundary electrophoresis. It appears to be indistinguishable in sedimentation coefficient and in electrophoretic mobility at *p*H 7.5 from carboxypeptidase A prepared by the procedure of Anson (1935).

The proteolytic coefficient of a solution of 5-times-crystallized enzyme using CGP as substrate was approximately 17[2]; the esterolytic activity measured with 0.1 M hippurylphenyllactate as substrate under conditions described by Bargetzi *et al.* (1963), was found to be 0.212 μmole of substrate hydrolyzed per minute per μg enzyme.[5] Activity toward substrates for chymotrypsin is the most frequently encountered contaminant of crystalline carboxypeptidase. Five-times-crystallized material prepared by the present procedure contained less than 0.005% chymotryptic activity (acetyl-L-tyrosine ethyl ester) and no detectable tryptic activity (benzoyl-L-arginine ethyl ester).

Carboxypeptidase A prepared by the present procedure is considerably more soluble in 1 M NaCl at neutral *p*H than is the enzyme isolated by the Anson procedure. Values as high as 23 mg protein per ml have been observed in this work and even higher values have been reported by Coleman *et al.* (1960) for the acetone powder enzyme, as compared to 3 mg per ml for the enzyme prepared by the method of Anson. The present enzyme resembles the protein prepared by Anson's procedure in gross chemical composition (Bargetzi *et al.*, 1963) and in the amino-terminal residues (Thompson, 1953; Coombs and Omote, 1962; Sampath Kumar *et al.*, 1963). Differences have been reported, however, in the properties of the respective apoenzymes following removal of metal. Vallee *et al.* (1960b) observed that the zinc atom could be removed from carboxypeptidase A with concomitant loss of enzymatic activity. This process was completely reversible when the enzyme prepared from acetone powder was used but only partially reversible with the Anson enzyme.

The use of acetone powders rather than fresh tissue as starting material offers several advantages in the isolation of carboxypeptidase A. The powder can be stored for long periods of time at low temperatures without loss of extractable procarboxypeptidase A. The zymogen can be extracted readily with water and activated under more controlled and reproducible conditions than those which obtain during tissue autolysis. Moreover, the yield compares favorably with that obtained by the earlier procedure. Anson reports a yield of 1 g crystalline enzyme from 1 liter of exudate collected from 10 kg wet tissue. By the present procedure, 1 g of crystalline enzyme was obtained from 500 g of powder, equivalent to 2.5 kg of wet tissue.

Although every attempt was made in the procedure described herein to control each preparative step from the extraction of the powder to the final crystallization, extensive trials over a period of several years revealed that some of the commercial powders were unsuitable for reasons which could be judged only operationally. Thus some powders seemingly contained excessive amounts of lipid material which sometimes prevented crystallization or yielded inactive crystals of the enzyme, coated with lipids. Other powders gave poor yields of crystalline enzyme. Sometimes pigment accompanied the enzyme through successive crystallizations and could not be completely removed by dialysis or by prolonged centrifugation. Failure to crystallize or to obtain fully active material even after repeated crystallizations and variable yields probably must all be ascribed to variations in the procedure attending or preceding the preparation of acetone powders. Among these, the history of the glands collected in packing houses is probably the single, least-controlled factor.

[5] The authors are grateful to Dr. Jean-Pierre Bargetzi for these data.

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