Mirelman, D., and Sharon, N. (1966), *Biochem. Biophys. Res. Commun. 24*, 237.
Muñoz, E., Ghuysen, J.-M., and Heymann, H. (1967), *Biochemistry 6*, 3659.
Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J. F., and Tinelli, R. (1966), *Biochemistry 5*, 3091.
Ou, L.-T., and Marquis, R. E. (1970), *J. Bacteriol. 101*, 92.
Petit, J. F., Adam, A., Witzerbin-Falszbin, J., Lederer, E., and Ghuysen, J.-M. (1969), *Biochem. Biophys. Res. Commun. 35*, 478.
Petit, J. F., Muñoz, E., and Ghuysen, J.-M. (1966), *Biochemistry 5*, 264.
Rudall, K. M., (1967) *in* Conformation of Biopolymers, Ramachandran, G. N., Ed., Vol. 2, New York, N. Y., Academic Press, p 751.
Schindler, C., and Schuhardt, V. T. (1966), *Proc. Nat. Acad. Sci. U. S. 51*, 414.
Sharon, N. (1964), *Proc. 3rd Symp. Fleming's Lysozyme, Milan*, 44/RT.
Shockman, G. D., Thompson, J. S., and Conover, M. J. (1967), *Biochemistry 6*, 1056.
Tipper, D. J. (1969), *Biochemistry 8*, 2192.
Tipper, D. J. (1971), *Int. J. System. Bacteriol.* (in press).
Tipper, D. J., and Berman, M. F. (1969), *Biochemistry 8*, 2183.
Tipper, D. J., Ghuysen, J.-M., and Strominger, J. L. (1965), *Biochemistry 4*, 468.
Tipper, D. J., and Strominger, J. L. (1966), *Biochem. Biophys. Res. Commun. 22*, 48.
Tipper, D. J., Strominger, J. L., and Ensign, J. C. (1967), *Biochemistry 6*, 906.
Tipper, D. J., Strominger, J. L., and Ghuysen, J.-M. (1964), *Science 146*, 781.
Van der Veen, J. M. (1963), *J. Org. Chem. 31*, 567.
Van Heijenoort, J., Elbaz, L., Dezelee, P., Petit, J. F., Bricas, E., and Ghuysen, J.-M. (1969), *Biochemistry 8*, 207.
Warth, A. D., and Strominger, J. L. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 528.

Isolation and Characterization of Carboxypeptidases A and B from Activated Pancreatic Juice*

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ABSTRACT: A procedure utilizing affinity chromatography as well as conventional techniques is outlined in detail for the efficient, large-scale purification of bovine carboxypeptidases A and B from activated pancreatic juice. Carboxypeptidase A thus prepared is in the β form of the enzyme. Three forms of carboxypeptidase B are found. The minor form, accounting for about 10% of the total enzyme, is a single-chain protein comparable to carboxypeptidase B isolated after tryptic activation of partially purified procarboxypeptidase B. The two predominant forms of carboxypeptidase B are two-chain proteins. The molecular weights of the component chains of each enzyme are 10,000 and 25,000. The amino acid compositions and enzymatic properties of the two-chain enzymes appear to be the same as that of the single-chain protein. A method of separating the chains of these proteins is also presented.

Studies of the structure and function of bovine carboxypeptidase B have been limited by the difficulties of its preparation. The standard procedure for the isolation of the enzyme involves the partial purification of the zymogen, procarboxypeptidase B, from pancreatic juice or extract (Wintersberger *et al.*, 1962; Kycia *et al.*, 1968). After activation by exogenous trypsin, carboxypeptidase B is crystallized. This procedure depends on the supply of unactivated pancreatic juice or extract and the prevention of activation during the preparation of the zymogen.

In view of the difficulties of avoiding spontaneous activation, we have developed an alternate route to the isolation of carboxypeptidase B from activated pancreatic secretions (Reeck *et al.*, 1971). The present paper describes the purification procedure in detail, as well as the characterization of the resulting enzymes. As a by-product of the present procedure, a method has also been developed for the isolation of pure carboxypeptidase A_β.

Experimental Procedure

Materials

DE-52 cellulose was obtained from Whatman Reeve Angel and Sephadex G-75 and Sepharose 4B from Pharmacia Fine Chemicals.

Bovine pancreatic juice was collected at the School of Veterinary Science, Washington State University, Pullman, Wash., using the general procedure of Keller *et al.* (1958). Despite antiseptic precautions and rapid freezing of the collected juice, the zymogens were fully activated on arrival at the University of Washington.

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Proteins used as standards in sodium dodecyl sulfate gel electrophoresis were all obtained from Worthington Biochemical Corp., except for myoglobin, which was obtained from Mann Research Laboratories. Bovine carboxypeptidase B (Wintersberger) was prepared by R. M. MacDonald of this laboratory by the method of Wintersberger *et al.* (1962). Soybean trypsin inhibitor (chromatographically prepared) was obtained from Worthington Biochemical Corp.

ϵ-Amino-*n*-caproyl-D-tryptophan was prepared from ϵ-amino-*n*-caproyl-D-tryptophan methyl ester by deesterification at pH 9.0 (0.1 M $NaHCO_3$) at room temperature. The reaction mixture was periodically adjusted to pH 9.0 with 6 M NaOH. The reaction, which was followed by high-voltage electrophoresis at pH 6.5, was essentially complete in 90 hr.

ϵ-Amino-*n*-caproyl-D-tryptophan methyl ester was synthesized according to the method of Horbett (1970). Sources of the materials used in the synthesis were as follows: carbobenzoxy-ϵ-amino-*n*-caproic acid, Cyclo Chemical Corp.; D-tryptophan methyl ester hydrochloride, Nutritional Biochemicals Corp.; and *N,N*-dicyclohexylcarbodiimide, Cyclo Chemical Corp. Guanidine hydrochloride (spectrophotometric grade) was obtained from Heico, Inc. β-Phenylpropionic acid, obtained from Eastman Organic Chemicals, was recrystallized from an ethanol–water mixture before use according to Pétra and Neurath (1969).

Sources of the substrates used for enzymatic assays were as follows: benzoylglycyl-L-arginine for kinetic measurements, Mann Research Laboratories; benzoylglycyl-L-arginine for routine assays and hippuryl-DL-phenylalanine, Fox Chemical Co.; benzoyl-L-tyrosine ethyl ester, Calbiochem; carbobenzoxyglycyl-L-phenylalanine, Eli Lilly and Co. and Cyclo Chemical Corp.; hippuryl-L-argininic acid was synthesized by R. L. Stevens of this laboratory following the procedure of Sanders (1970); and *p*-tosyl-L-arginine methyl ester, Cyclo Chemical Corp.

Methods

Amino Acid Composition. A Spinco Model 120 amino acid analyzer was used for all analyses. The amino acid compositions were derived from analyses of protein samples which had been hydrolyzed in 6 N HCl at 110° for 24, 48, 72, and 96 hr. Individual analyses were placed on a common molar basis by adjusting all alanine values to a constant number. The reported values for serine and threonine are extrapolations to zero time of hydrolysis assuming first-order kinetics of destruction. Isoleucine and valine values are the average of 96-hr hydrolyses unless these amino acids were fully liberated after 72 hr of hydrolysis, in which case the 72- and 96-hr results were averaged and reported. Half-cystine and methionine were determined after performic acid oxidation as cysteic acid and methionine sulfone (Hirs, 1967). Tryptophan was determined by the method of Barman and Koshland (1967).

Amino-Terminal Sequence Determinations. A Beckman Sequencer, designed on the general principles of Edman and Begg's protein sequenator (Edman and Begg, 1967), was used for the determination of the amino-terminal sequences of proteins and polypeptides. A volatile buffer system was employed, and 1,4-butanedithiol was added to the chlorobutane to stabilize the sequenator products (Hermodson *et al.*, 1970). The phenylthiohydantoin amino acids were identified by gas chromatography, with the exceptions of histidine and arginine which were identified by spot tests using diazotized *p*-anisidine (Sanger and Tuppy, 1951) and phenanthrenequinone (J. Ohms, personal communication), respectively. Complete details of the sequenator methodology as developed in this laboratory will be published elsewhere (M. A. Hermodson, unpublished).

Analytical Polyacrylamide Disc Gel Electrophoresis. Proteins were subjected to polyacrylamide disc gel electrophoresis at pH 8.3 following the general method of Davis (1964). This method was slightly altered in that the separating gels were poured before the sample gels and the concentrating gel was omitted since its inclusion did not affect the resulting electrophoresis. Gels were run at room temperature in an apparatus purchased from Hoeffer Scientific Instruments. Distilled water at 4° was used for the preparation of the reservoir buffer. Gels were stained with 1% amido schwarz in 7% acetic acid for at least 1 hr. They were destained using the Quick Gel Destainer of the Canalco Co.

Column Chromatography and Gel Filtration. All columns were developed in the cold room at 4°. Columns were packed with media as directed by the manufacturers. Ion-exchange cellulose and Sephadex were stored in 0.03% toluene to prevent microbial growth.

Dialysis Tubing Preparation. Visking dialysis tubing was cut into appropriate lengths and immersed in approximately 0.1 M $NaHCO_3$. The tubing was heated on a steam cone for 8 hr and the water replaced with a fresh solution of $NaHCO_3$. After heating for another 8 hr, the tubing was rinsed several times with distilled water and stored for later usage in 0.03% toluene.

Enyzmatic Assays. p-Tosyl-L-arginine methyl ester was employed to assay spectrophotometrically for trypsin activity (Hummel, 1959). *N*-Benzoyl-L-tyrosine ethyl ester was the substrate for the sptectrophotometric assay for chymotrypsin activity (Hummel, 1959) except that the substrate was dissolved directly in 0.04 M Tris, pH 7.8, containing 0.05 M $CaCl_2$, by heating and shaking vigorously (Horbett, 1970). No methanol was added to dissolve the substrate.

Exopeptidase activities were routinely determined with carbobenzoxyglycyl-L-phenylalanine and benzoylglycyl-L-arginine as substrates for carboxypeptidases A and B, respectively. The hydrolysis of carbobenzoxyglycyl-L-phenylalanine was followed by measuring the decrease in absorbance at 225 nm of a 1×10^{-3} M solution dissolved in 0.005 M Tris, pH 7.5, containing 0.10 M NaCl (Whitaker *et al.*, 1966). Activity toward benzoylglycyl-L-arginine was measured in a spectrophotometric assay as described by Folk *et al.* (1960).

The activities of carboxypeptidases B toward hippuryl-L-argininic acid were determined by the spectrophotometric assay of Folk and Gladner (1959) using 0.025 M Tris, pH 7.65, containing 0.1 M NaCl, as the solvent for the substrate.

The hydrolysis of hippuryl-DL-phenyllactic acid was followed by the increase in absorbance at 254 nm (McClure *et al.*, 1964). Substrate was dissolved in 0.005 M Tris, pH 7.5, containing 0.10 M NaCl.

Routine spectrophotometric assays were performed with the Gilford Model 2000 spectrophotometer. A Cary Model 16 spectrophotometer was used for kinetic analyses and determinations of specific activities.

Sodium Dodecyl Sulfate Gel Electrophoresis. The general procedures of Weber and Osborn (1969) were used with the following modifications. The protein samples were incubated for 2 hr at 37° in 0.01 M sodium phosphate, pH 7.0, containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. This modification was prompted by the finding that carboxypeptidase B from activated pancreatic juice apparently did not enter gels when electrophoresis was conducted after the incubation suggested by Weber and Osborn. The same enzyme

did, however, run well in electrophoresis after the modified incubation. The amount of brom phenol blue suggested by Weber and Osborn (1969) was increased tenfold for adequate visualization of the dye band.

Preparation of Modified Gels for Affinity Chromatography. The procedures used for activation of Sepharose 4B with cyanogen bromide and the covalent coupling to the activated Sepharose of an enzyme inhibitor (soybean trypsin inhibitor or ϵ-amino-*n*-caproyl-D-tryptophan) were those developed by Porath and coworkers (Axén *et al.*, 1967; Porath *et al.*, 1967) and by Cuatrecasas *et al.* (1968). The design of Horbett (1970) was used for the reaction vessel to activate Sepharose. The coupling reaction was performed at pH 9.5 (0.1 M $NaHCO_3$). Using 20 mg of soybean trypsin inhibitor per ml of Sepharose, about 65% of the protein was permanently attached to the gel after the coupling reaction. The coupling mixture for ϵ-amino-*n*-caproyl-D-tryptophan contained 7.3 mmoles of the inhibitor and 200 ml of activated Sepharose. Twenty per cent (1.5 mmoles) of the inhibitor could not be removed from the gel after the coupling reaction by extensive washings with 0.05 M Tris, pH 7.5, and 0.1 M sodium acetate, pH 3.0. Thus the concentration of inhibitor bound to the Sepharose was about 7.5 mM.

Kinetic Measurements with Benzoylglycyl-L-arginine. Three separate velocity measurements were made at seven substrate concentrations. For each measurement 50 μl of the enzyme [0.93×10^{-2} mg ml^{-1} for carboxypeptidase B II and 1.02×10^{-1} mg ml^{-1} for carboxypeptidase B (Wintersberger)] was added to 3.0 ml of the substrate. The enzymes were stored at 4° in 0.01 M Tris, pH 8.0, containing 0.1 M NaCl. The solvent for the substrate was 0.025 M Tris, pH 7.65, containing 0.1 M NaCl. All measurements were made at 25°.

The data were plotted according to Eadie and the slope and intercept of the best-fitting straight line determined by a least-squares program using an Olivetti-Underwood Programma 101. The reported errors in K_m and k_0 are estimated standard errors of the slope and intercept values.

Ultracentrifugation. High-speed sedimentation equilibrium analyses were performed using the six-channel centerpiece of Yphantis (1964). The optimum speed for centrifugation was estimated by eq 12 of Teller *et al.* (1969), and the sample was centrifuged for 20 hr to achieve sedimentation equilibrium. Photographs of the Rayleigh interference patterns at equilibrium were taken using Kodak II-G photographic plates. Baseline interference patterns were photographed at 3200 rpm after photographs were taken of the equilibrium pattern and the cell was thoroughly shaken. Fringe displacement was measured as a function of position in the cell with a microcomparator.

The data were analyzed by a computer program of Teller and collaborators (Teller *et al.*, 1969), which calculates the molecular weight averages, M_n, M_w, and M_z, as functions of protein concentrations as well as whole-cell averge molecular weights. The solvent density was determined by pycnometry. Carboxypeptidases B I and II had partial specific volumes of 0.730 and 0.726, respectively, as calculated from their amino acid compositions.

Zinc Analyses. A single-element zinc lamp (Perkin-Elmer) was used in the Model 303 atomic absorption spectrophotometer of the same company. All samples were dialyzed prior to measurement in dialysis tubing which was pretreated to remove impurities (Hughes and Klotz, 1956). In some cases glass-distilled water was used for all solutions. It was found, however, that the stock supply of distilled water contained no detectable zinc and was thus suitable for use in zinc analyses. Standard zinc solutions for calibration of the instrument were prepared by dissolving analytical reagent grade zinc metal in a minimum amount of concentrated HCl and diluting with distilled water to the appropriate volume.

Results

Preparative Procedures. The removal of trypsin and chymotrypsin from the pancreatic juice proved to be necessary because these enzymes were somewhat unexpectedly found to adhere to the ϵ-amino-*n*-caproyl-D-tryptophan–Sepharose column used to separate carboxypeptidases A and B. Previous experiments in this laboratory had indicated that an affinity column of soybean trypsin inhibitor coupled to Sepharose might well be effective in this capacity (Robinson *et al.*, 1971).

CHROMATOGRAPHY ON SOYBEAN TRYPSIN INHIBITOR–SEPHAROSE. For preparative work a 5 × 35 cm column of Sepharose with 7 g of soybean trypsin inhibitor attached was used at a flow rate of 600 ml hr^{-1} maintained by a Technicon pump. After removing a white precipitate by centrifugation,[1] 1600 ml of active bovine juice was applied to the column and the column was equilibrated with 0.02 M Tris, pH 7.5, containing 0.5 M NaCl.

After application of the juice to the column, the starting buffer (0.02 M Tris, pH 7.5, containing 0.5 M NaCl) was pumped through the column for ~3 hr to elute any protein not specifically adsorbed. As can be seen in Figure 1, both carboxypeptidases A and B were eluted under these conditions, although they were somewhat retarded when the juice itself was applied to the column. If a buffer containing a lower concentration of NaCl was used (*e.g.*, 0.05 M), the carboxypeptidases were eluted, but in a much larger volume. Apparently the higher salt concentration is needed to weaken effectively the binding of the carboxypeptidases to the column.

After the absorbance of the effluent had returned to nearly zero, 0.1 M sodium acetate, pH 3.0, was applied, resulting in the elution of trypsin and chymotrypsin activities from the column. Activities were recovered as follows: carboxypeptidase B (benzoylglycyl-L-arginine) 95%; carboxypeptidase A (carbobenzoxyglycyl-L-phenylalanine) 75%; trypsin (tosyl-L-arginine methyl ester) 70%; and chymotrypsin (benzoyl-L-tyrosine ethyl ester) 90%.

No trypsin activity was detected in the carboxypeptidase fraction of the effluent, and the chymotrypsin activity relative to carboxypeptidase B activity was only 0.4% as great as in the unfractionated juice. The carboxypeptidase fraction was concentrated by ultrafiltration to 210 ml using a UM-10 membrane, which would allow any polypeptides of molecular weight less than 10,000 to pass through the membrane. Of the 280-nm-absorbing material, 73% did pass through the membrane, but none of the carboxypeptidase activity did. The low molecular weight material must constitute proteolysis products of the pancreatic zymogens and enzymes because in inactive pancreatic juice 75% of the proteins are known to be either proteolytic zymogens or nucleolytic enzymes, all of which have molecular weights over 10,000 (Keller *et al.*, 1958).

Carboxypeptidase A could be selectively precipitated from

[1] This precipitate was partially soluble in 5 M NaCl. That portion of the precipitate which was soluble in 5 M NaCl accounted for about 7% of the total protein in 1600 ml of juice and contained about 2% of the total carboxypeptidase A activity, 0.3% of the total chymotrypsin activity, and none of the trypsin or carboxypeptidase B activities.

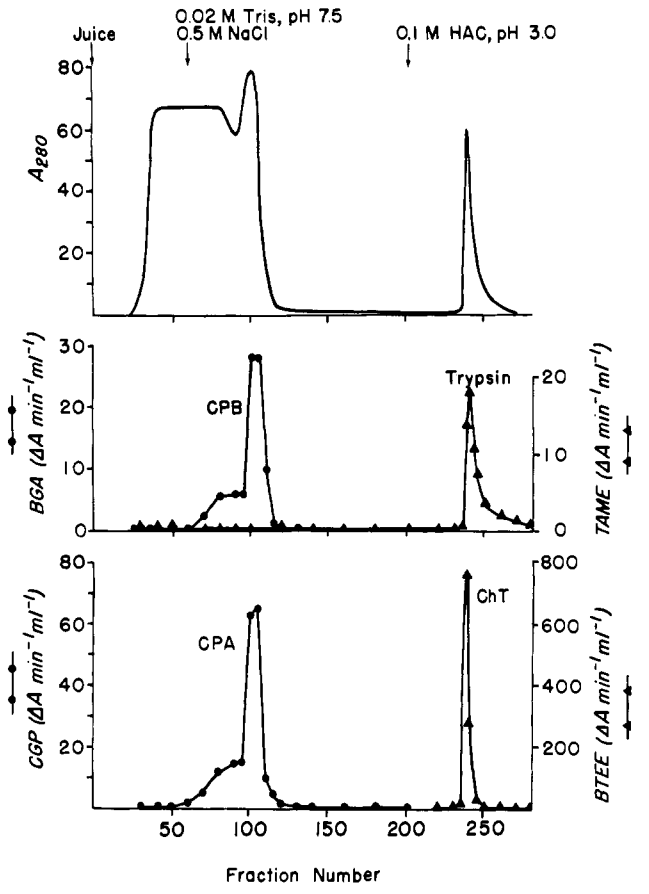

FIGURE 1: Chromatography of activated pancreatic juice on soybean trypsin inhibitor–Sepharose. Fraction volume was 25 ml. Other details are given in the text.

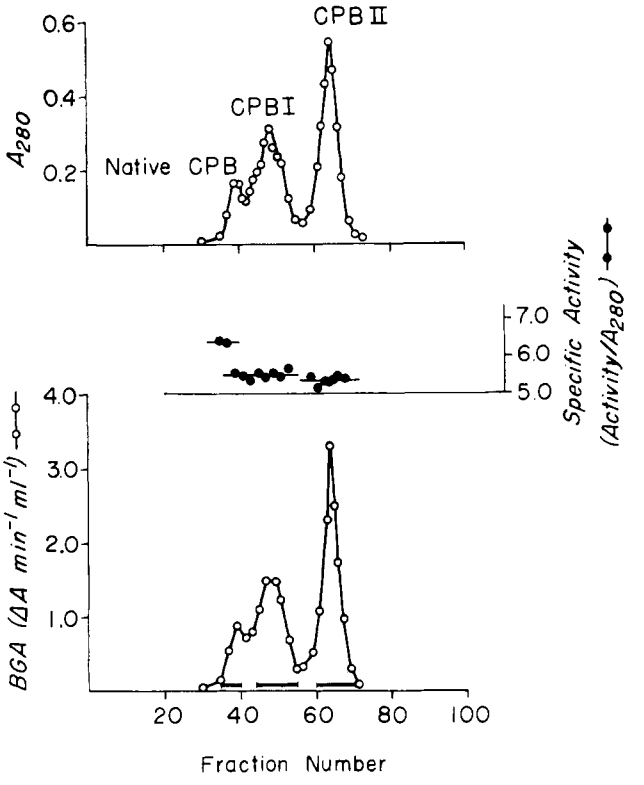

FIGURE 2: Chromatography of carboxypeptidase B from activated pancreatic juice on DE-52 cellulose. The fraction volume was 25 ml. Other details are given in the text.

the concentrated sample by dialysis for 36 hr against a large excess of 0.02 M Tris, pH 7.5. Most of the carboxypeptidase A activity (91%) precipitated during this dialysis, but only 1% of the carboxypeptidase B activity precipitated. This removal of carboxypeptidase A allowed the chromatography of larger samples of carboxypeptidase B on the next column which adsorbs both carboxypeptidases.

The supernatant of the previous step was pumped onto a 2.5 × 35 cm column of Trp-Sepharose which had been equilibrated with 0.02 M Tris, pH 7.5, containing 0.05 M NaCl. The elution profile and activity measurements were given previously (Reeck *et al.*, 1971). After the elution of an inactive breakthrough peak, 0.15 M NaCl in 0.02 M Tris, pH 7.5, was applied to elute carboxypeptidase B. After the carboxypeptidase B activity was nearly completely eluted, 0.5 M NaCl was applied to elute carboxypeptidase A.

In its essential features this chromatography was entirely reproducible. In some preparations, however, an inactive protein peak emerged after the breakthrough and before the application of 0.15 M NaCl. Sometimes an inactive peak was observed after the elution of carboxypeptidase B but before 0.5 M NaCl was applied. The nature of these proteins is unknown and no proven explanation can be offered for their adsorption by the Trp-Sepharose.

The portion of the effluent which contained carboxypeptidase B was concentrated by ultrafiltration before chromatography on DE-52 cellulose.

CHROMATOGRAPHY ON DE-52 CELLULOSE. The carboxypeptidase B sample from the preceding chromatography was dialyzed overnight at a protein concentration not exceeding 0.5 mg ml^{-1} against 0.02 M Tris, pH 8.0, and then applied to a 2.5 × 40 cm column of DE-52 cellulose which had been equilibrated with the same buffer containing 10^{-4} M $ZnCl_2$. The column was developed after application of the sample by use of a linear salt gradient from 0.0 to 0.1 M NaCl in a total volume of 2000 ml of 0.02 M Tris, pH 8.0, containing 10^{-4} M $ZnCl_2$. The elution profile and assays are shown in Figure 2, which reveals three peaks of carboxypeptidase B labeled in order of their elution, native carboxypeptidase B, carboxypeptidase B I,[2] and carboxypeptidase B II. In some preparations a small amount of material was observed as a breakthrough peak and in all preparations some reddish pigment adhered to the upper portion of the column and was not eluted during the chromatography.

Inclusion of zinc in the elution buffer was necessary in order to stabilize the enzymatic activity. Without added zinc, the specific activities of carboxypeptidases B were higher than that reported for purified carboxypeptidase B (Wintersberger *et al.*, 1962). Furthermore, the specific activities of the various fractions declined over the course of several days to

[2] Carboxypeptidase B I appears to be heterogeneous chromatographically. Nonetheless, the specific activities of the various fractions in this peak are essentially constant, indicating that the heterogeneity must be due to multiple forms of carboxypeptidase B. Furthermore, carboxypeptidase B I appears to be homogeneous on polyacrylamide disc gel electrophoresis (Reeck *et al.*, 1971). No heterogeneity has been observed by sequenator analysis in the light chain of carboxypeptidase B I. We have, however, observed a significant amount (10–15%) of a second sequence in the heavy-chain analysis. Specifically this sequence corresponds to the sequence starting with the third residue (Glu) of the major sequence. This species presumably accounts for the observed chromatographic heterogeneity of carboxypeptidase B I.

TABLE I: Purification of Bovine Carboxypeptidase B.

Preparative Step	Volume (ml)	Enzyme Concn (ΔA min^{-1} ml^{-1})	Total Units (ΔA min^{-1})	Protein[a] (mg ml^{-1})	Specific Activity (ΔA min^{-1} mg^{-1})	Yield (%)	Purification (-fold)
Pancreatic juice	1600	0.784	1254	3.87	0.097	100	1.0
Carboxypeptidase fraction from soybean trypsin inhibitor–Sepharose column (concentrated by ultrafiltration)	210	5.51	1157	5.86	0.448	92	4.6
Supernatant from dialysis against 0.02 M Tris, pH 7.5	230	4.62	1063	1.21	1.82	85	21
Sample for DE-52 column	73	13.4	978	1.37	4.65	78	48
Carboxypeptidase B fractions from DE-52 column	1000	0.889	889	0.078	5.45	71	56

[a] Protein concentration was determined by absorbance at 280 nm assuming $A_{1cm}^{0.1\%} = 2.1$ (Cox *et al.*, 1962).

approximately that given by Wintersberger *et al.* (1962). The specific activities were constant when zinc was included in the elution buffer.

The chromatogram shown in Figure 2 differs from that reported previously (Reeck *et al.*, 1971) in having a carboxypeptidase B peak which emerges before carboxypeptidase B I. This earlier peak, termed "native carboxypeptidase B" for reasons which will be apparent, has been consistently observed in many preparations since the early work was reported (Reeck *et al.*, 1971).

A summary of the purification of bovine carboxypeptidase B from activated pancreatic juice is presented in Table I. The overall recovery of the enzyme was 71%, and from 1600 ml of juice 78 mg of pure carboxypeptidase B was prepared.

Characterization of Carboxypeptidase A Isolated from Activated Pancreatic Juice. The protein which precipitated during dialysis of the carboxypeptidase fraction from the soybean trypsin inhibitor–Sepharose column was dissolved in 10% LiCl and crystallized by dialysis against distilled water as described by Anson (1937). Chromatography of the crystalline enzyme on DE-52 cellulose according to the method of Pétra and Neurath (1969) resulted in the elution of a single symmetrical peak in a position corresponding to carboxypeptidase A_β^{Val} (Figure 3).

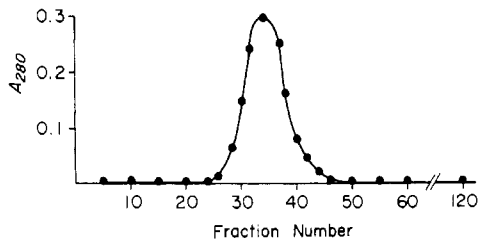

FIGURE 3: Chromatography of carboxypeptidase A from activated pancreatic juice on DE-52 cellulose. The chromatographic system is described by Pétra and Neurath (1969). The column was developed by applying 0.05 M Tris, pH 7.5, containing 0.005 M β-phenylpropionate, and 0.04 M LiCl, at a flow rate of 39 ml/hr to a 1.5 × 100 cm column. The fraction volume was 13 ml. The protein emerged at an elution volume which corresponded to that where carboxypeptidase A_β^{Val} was observed to elute in chromatographies performed at nearly the same time on heterogeneous carboxypeptidase A. The chromatography was continued until 1600 ml of buffer had eluted.

The fact that carboxypeptidase A isolated from activated pancreatic juice was purely in the β form was also demonstrated by sequenator analysis of the crystalline material prior to chromatography. The sequenator data, outlined in Table II, ruled out the presence of significant amounts of either the α or γ forms of the enzyme. The amino acids identified after the second Edman degradation (turn 2) are particularly important in this regard. Both arginine and tyrosine, which are expected to be released from the α and γ forms of carboxypeptidase A by the second Edman degradation, can be detected in very low amounts, yet neither was observed at turn 2. The sequence of major amino acids observed at each analysis, constituting about 95% of the total amount of amino acids observed at each turn, corresponded to those expected from carboxypeptidase A_β.

The sequence Ala-Gly-Ala-X-Ser-X-Pro could be constructed from the minor amino acids detected in the turns analyzed. This sequence can be found in carboxypeptidase A_α, starting with residue 154. Furthermore, this sequence is preceded by a lysyl residue, which suggests that a small percentage, about 5%, of the carboxypeptidase A molecules had undergone hydrolysis of peptide bond Lys_{153}-Ala_{154},

TABLE II: Sequenator Analysis of Carboxypeptidase A from Active Pancreatic Juice.

Turn	Major Amino Acid[a]	Minor Amino Acids
1	Ser	Ala
2	Thr	Gly, Val
3	Asn	Ala, Ile
5	Phe	Ser
7	Tyr	Pro, Phe, Ile

[a] In each turn, the major amino acid constituted about 95% of the total amount of amino acids observed. The products of turns 4 and 6 were not analyzed.

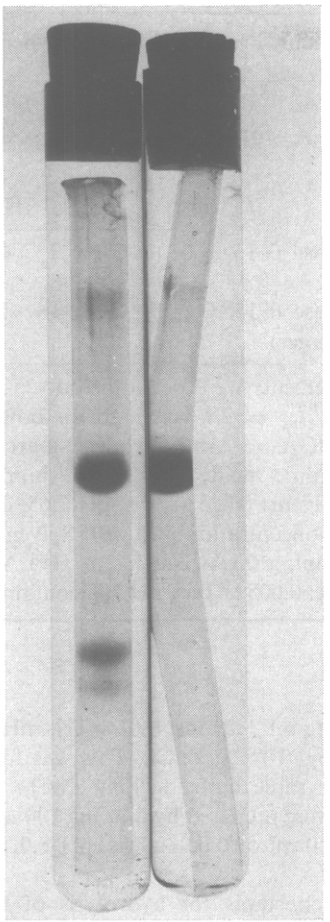

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of carboxypeptidase A from activated pancreatic juice. Migration was from top to bottom (cathode to anode). Left: carboxypeptidase A before chromatography on DE-52 cellulose (Figure 3). Right: carboxypeptidase A after chromatography on DE-52 cellulose. Standard proteins used in the molecular weight determinations were bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, and ribonuclease.

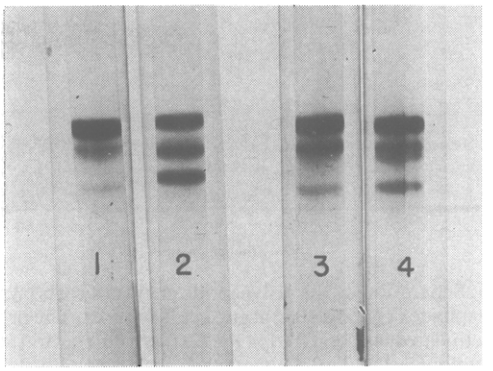

FIGURE 5: Polyacrylamide disc gel electrophoresis. Migration was from top to bottom (cathode to anode). The gels were severed at the dye front before staining. Two similar experiments are illustrated; in the first experiment, tube 1, containing 35 μg of carboxypeptidase B (Wintersberger), is compared with tube 2, a mixture of 20 μg of carboxypeptidase B (Wintersberger) and 15 μg of the product of the Trp-Sepharose column; in the second experiment, tube 3 (a control, identical with tube 1), is compared with tube 4, a mixture of 35 μg of carboxypeptidase B (Wintersberger) and 15 μg of the product of the Trp-Sepharose column. Carboxypeptidase B II appears to correspond in mobility to the fastest migrating component of carboxypeptidase B (Wintersberger). From tube 4 it does not appear that carboxypeptidase B I corresponds to the second minor component of carboxypeptidase B (Wintersberger). The particular preparation of carboxypeptidase B from activated pancreatic juice used in these gels contained none of the species which emerges before carboxypeptidase B I on DE-52 cellulose chromatography (see Figure 4).

presumably catalyzed by trypsin. Examination of the three-dimensional model of Lipscomb and coworkers (1968) elevaled that this bond is located on the surface of the molecule and could well be susceptible to proteolysis.

The results from sodium dodecyl sulfate gel electrophoresis are consistent with the hydrolysis of this bond. As can be seen from Figure 4, the crystalline carboxypeptidase A which had not been chromatographed on DE-52 cellulose displayed a major band corresponding to a molecular weight of 34,000 and minor bands corresponding to molecular weights of 18,000 and 15,000. The latter are not seen when the chromatographed enzyme is subjected to sodium dodecyl sulfate gel electrophoresis.

Characterization of the Multiple Forms of Carboxypeptidase B from Activated Pancreatic Juice. GEL ELECTROPHORESIS. As was previously shown (Reeck *et al.*, 1971), carboxypeptidases B I and II are essentially pure as judged by analytical polyacrylamide disc gel electrophoresis. Furthermore, as is shown in Figure 5, each is different from the main component of the carboxypeptidase B prepared by crystallization of the tryptic activation product of partially purified procarboxypeptidase B, termed carboxypeptidase B (Wintersberger). Carboxypeptidase B (Wintersberger) appears to contain a small amount of carboxypeptidase B II (the most anionic component), but none of carboxypeptidase B I. "Native carboxypeptidase B" from the chromatography shown in Figure 2 corresponds in this disc gel system to the main component of carboxypeptidase B (Wintersberger), but also contains substantial amounts of carboxypeptidase B I.

Examination of carboxypeptidases B I and II by sodium dodecyl sulfate gel electrophoresis revealed that each enzyme is composed of two polypeptide chains having approximate weights of 10,000 and 25,000 (Reeck *et al.*, 1971). Native carboxypeptidase B from chromatography on DE-52 cellulose produces a main band on sodium dodecyl sulfate gel electrophoresis corresponding to a molecular weight of 34,000 and fainter bands corresponding to molecular weights of 25,000 and 10,000. It thus appears that native carboxypeptidase B from activated pancreatic juice is contaminated with carboxypeptidase B I, but consists mostly of a protein comparable to carboxypeptidase B (Wintersberger), a single-chain protein whose molecular weight was determined to be 34,000 by sodium dodecyl sulfate gel electrophoresis (Reeck *et al.*, 1971).

MOLECULAR WEIGHTS OF CARBOXYPEPTIDASES B I AND B II. Whole-cell weight-average molecular weights, determined at three protein concentrations and two different temperatures (4 and 20°), yielded a value of 35,200 ± 1200 for carboxypeptidase B I and 36,100 ± 900 for carboxypeptidase B II. The average of all 12 determinations was 35,700 ± 1000 and was considered to be representative of both proteins.

AMINO ACID COMPOSITIONS OF CARBOXYPEPTIDASES B I AND B II. The amino acid compositions of carboxypeptidases B I and II and carboxypeptidase B (Wintersberger) are presented in Table III. The compositions of the two-chain forms of carboxypeptidase B are the result of single analyses after 24, 48, 72, and 96 hr of hydrolysis and were derived as described

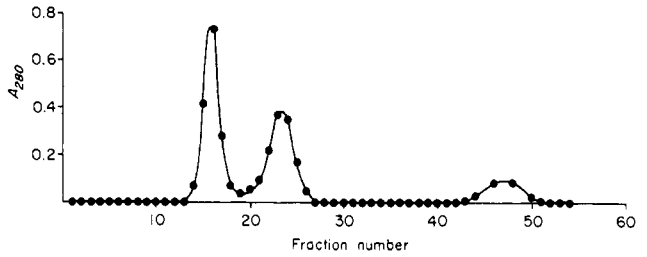

FIGURE 6: Separation of the polypeptide chains of carboxypeptidase B II on Sephadex G-75 in 10% acetic acid–4 M urea. The protein was S-aminoethylated, as described in the text, prior to gel filtration. The fraction volume was 13 ml. Other details are given in the text.

in Methods. The compositions of the two-chain enzymes from activated pancreatic juice are very similar to that of carboxypeptidase B (Wintersberger).

ZINC BINDING MEASUREMENTS. Samples of carboxypeptidases B I and II were dialyzed extensively against zinc-free buffer, 0.001 M Tris, pH 8.0, containing 0.02 M NaCl, and the amount of zinc bound to the proteins after dialysis was measured by atomic absorption spectrophotometry. (Zinc had been present in excess before dialysis because of its inclusion in the elution buffer for the DE-52 chromatography (Figure 2)). Two separate measurements for each enzyme gave the following results, expressed as gram-atoms of zinc per mole of protein: carboxypeptidase B I: 0.99, 1.05; carboxypeptidase B II: 1.04, 1.01. Thus each protein is capable of tightly binding one zinc atom per molecule.

ENZYMATIC PROPERTIES OF CARBOXYPEPTIDASE B II AND CARBOXYPEPTIDASE B (WINTERSBERGER). Carboxypeptidases B (Wintersberger) and B II were dialyzed at a protein concentration of 0.5 mg ml^{-1} against 0.01 M Tris, pH 8.0, containing 0.10 M NaCl and 10^{-4} M $ZnCl_2$. This was followed by dialysis against the same buffer lacking $ZnCl_2$. Stock solutions of the enzyme were prepared by diluting 100 μl of the dialyzed samples with 5.0 ml of 0.01 M Tris, pH 8.0, containing 0.10 M NaCl.

TABLE III: Amino Acid Compositions of Bovine Carboxypeptidases B.

	Carboxypeptidase B I[c]	Carboxypeptidase B II[c]	Carboxypeptidase B (Wintersberger)[a,c]
Asp	29.7	28.3	26.6
Thr	28	28	26.6
Ser	28	28	26.6
Glu	26.2	27.1	24.3
Pro	12.7	11.7	12.5
Gly	23.4	22.4	22.0
Ala	24.0	23.4	22.3
Cys/2	7.64	7.51	7.00
Val	15.5	15.3	14.1
Met	5.13	6.47	6.18
Ile	15.4	15.1	16.2
Leu	20.5	20.6	20.8
Tyr	19.7	20.1	22.4
Phe	12.7	12.6	12.1
Trp[b]	9.7	9.6	10.2
Lys	16.9	17.0	17.2
His	4.78	6.34	7.21
Arg	11.3	11.5	13.5

[a] Data from Cox *et al.* (1962). [b] Determined by the method of Barman and Koshland (1967). [c] Residues/35,000 daltons.

TABLE IV: Enzymatic Properties of Carboxypeptidases B.[a]

Enzyme	Kinetic Constants for BGA Hydrolysis[b] K_m (mM)	k_0 (sec^{-1})	Specific Activities (μmoles min^{-1} mg^{-1}) HAA[c]	HPLA[d]
Carboxypeptidase B II	0.15 ± 0.01	57 ± 2	250	49
Carboxypeptidase B (Wintersberger)	0.15 ± 0.01	53 ± 1	230	42

[a] All measurements were performed at 25°. An extinction coefficient of $A_{1cm}^{0.1\%} = 2.1$ was used for both enzymes (Cox *et al.*, 1962). BGA = *N*-benzoylglycyl-L-arginine; HAA = hippuryl-L-argininic acid; HPLA = hippuryl-DL-phenyllactic acid. [b] Solvent: 0.025 M Tris, pH 7.65, containing 0.1 M NaCl. [c] HAA concentration: 1.0 mM. Solvent: 0.025 M Tris, pH 7.65, containing 0.1 M NaCl. [d] DL-HPLA concentration: 1.0 mM. Solvent: 0.005 M Tris, pH 7.5, containing 0.1 M NaCl.

The kinetic constants for hydrolysis of benzoylglycyl-L-arginine and specific activities of the two enzymes toward hippuryl-DL-phenyllactic acid and hippuryl-L-argininic acid are summarized in Table IV. There appears to be no significant difference between the two enzymes in any of these enzymatic properties.

SEQUENATOR ANALYSIS OF CARBOXYPEPTIDASE B (WINTERSBERGER). The amino-terminal sequence of native carboxypeptidase B (Wintersberger) was determined using a sequenator. A single amino acid was observed after each of ten cycles of the Edman degradation, and the sequence of these amino acids corresponded to the sequence of the light chains of carboxypeptidases B I and II previously reported (Reeck *et al.*, 1971). This finding provides an unambiguous, chemical confirmation that the light chain of carboxypeptidases B I and II corresponds to the amino-terminal region of the intact enzyme.

Separation and Characterization of the Polypeptide Chains of Carboxypeptidases B I and B II. SEPHADEX G-75 GEL FILTRATION. Carboxypeptidase B I or II was reduced and S-aminoethylated with ethylenimine as described by Cole (1967) and the sample applied directly to a 2.5 × 110 cm column of Sephadex G-75 equilibrated with 10% acetic acid containing 4 M urea. The protein elution profile is shown in Figure 6. Precisely the same profile was obtained for both enzymes. Furthermore, the first two peaks were also observed if the enzymes were not reduced and S-aminoethylated but simply adjusted to the solvent conditions of the column and applied to the column. It follows, therefore, that the two chains of each protein are not held together by disulfide bonds. The elution of the third peak shown in Figure 6 required the total volume of the column; thus this material constitutes low molecular weight compounds. The third peak is not observed if the protein is not S-aminoethylated.

Sodium dodecyl sulfate gel electrophoresis confirmed that

TABLE V: Amino Acid Compositions of the Separated Chains of Carboxypeptidases B I and II.[a]

	Carboxypeptidase B I				Carboxypeptidase B II			
	Light Chain (Residues/ 10,000 Daltons)	Heavy Chain (Residues/ 25,000 Daltons)	Sum	Intact Protein[b] (Residues/ 35,000 Daltons)	Light Chain (Residues/ 10,000 Daltons)	Heavy Chain (Residues/ 25,000 Daltons)	Sum	Intact Protein[b] (Residues/ 35,000 Daltons)
Asp	7.49	23.1	30.6	29.7	7.37	22.3	29.7	28.3
Thr	8.3	20	28	28	7.8	19	27	28
Ser	6.7	21	28	28	6.3	20	26	28
Glu	9.42	16.5	25.9	26.2	9.46	16.9	26.4	27.1
Pro	3.82	8.31	12.1	12.7	4.09	8.28	12.4	11.7
Gly	6.59	16.5	23.1	23.4	6.66	15.9	22.6	22.4
Ala	7.39	16.5	23.9	24.0	7.41	15.7	23.1	23.4
Cys/2	1.90	5.22	7.12	7.64	1.98	4.92	6.90	7.51
Val	5.40	10.0	15.4	15.5	5.38	9.67	15.1	15.3
Met	1.03	4.34	5.37	5.13	0.89	4.61	5.50	6.47
Ile	5.57	9.47	15.0	15.4	4.55	10.4	15.0	15.1
Leu	5.13	16.3	21.4	20.5	3.91	17.1	21.0	20.6
Tyr	2.31	15.7	18.0	19.7	2.37	15.9	18.3	20.1
Phe	4.88	7.30	12.2	12.7	4.96	6.80	11.8	12.6
Lys	4.30	14.3	18.6	16.9	4.26	14.7	19.0	17.0
His	2.05	4.17	6.22	4.78	1.96	5.31	7.27	6.34
Arg	3.18	7.85	12.0	11.3	4.19	8.27	12.5	11.5
Trp	(4)	(6)	(10)	9.7	(4)	(6)	(10)	9.6

[a] Since four tryptophans have been placed in the light chains (Reeck *et al.*, 1971), it is assumed that the remaining six are in the heavy chains. [b] From Table III.

the first peak eluting from the gel filtration column was the heavy chain of carboxypeptidase B and the second peak the light chain.

AMINO ACID COMPOSITION OF THE SEPARATED CHAINS. For the determination of their amino acid compositions, the light and heavy chains of carboxypeptidases B I and II were prepared by gel filtration of the unmodified proteins. In Table V are presented the amino acid compositions, determined as described in Methods using duplicate samples at each time of hydrolysis. Summation of the compositions of the light and heavy chains of each protein agrees well with the compositions of intact carboxypeptidases B I and II, respectively.

Discussion

Carboxypeptidase A has been available in large quantities by application of any of several previously developed purification procedures (Anson, 1937; Putnam and Neurath, 1946; Cox *et al.*, 1964; Allan *et al.*, 1964). More recently the problem of heterogeneity of the enzyme from these preparations has been resolved by ion-exchange chromatography (Pétra and Neurath, 1969; Pétra *et al.*, 1969). Nonetheless the present method of preparation may offer certain advantages over the previous procedures. By use of the soybean trypsin inhibitor–Sepharose column one may obtain a single preparation of approximately 200 mg of carboxypeptidase A; the soybean trypsin inhibitor–Sepharose column can be developed in a few hours, and the resulting enzyme fraction requires only concentration by ultrafiltration (overnight) and precipitation by dialysis for 36 hr. The latter two steps involve little actual manipulation while they proceed.

Except for possible genetic variation (Walsh *et al.*, 1966) the product of this procedure is practically homogeneous as judged by chromatography on DE-52 cellulose, sodium dodecyl sulfate gel electrophoresis, and sequenator analysis. It corresponds to the β form of carboxypeptidase A. In the particular preparation illustrated in Figure 3, the β enzyme is apparently the "Val" allotype.

The present purification scheme is capable of producing in an efficient manner relatively large quantities of bovine carboxypeptidase B of high purity as well. The procedure is particularly well suited to large-scale purification because of the simplicity of the first two chromatographic steps, each of which utilizes an enzyme inhibitor covalently bound to Sepharose 4B. As pointed out by Cuatrecasas *et al.* (1968), Sepharose has excellent flow properties. In this work, the soybean tyrpsin inhibitor–Sepharose column (5 × 35 cm) was pumped at a flow rate of 600 ml hr^{-1} and the Trp-Sepharose column (2.5 × 35 cm) at 100 ml hr^{-1}. In actuality each column could be pumped considerably faster. In addition, each of these columns is easily regenerated for repeated usage; no repacking of either column is necessary.

The present purification scheme could be extended to produce α and β trypsins from the juice used for purification of carboxypeptidases. Robinson *et al.* (1971) have developed a chromatographic system for the separation of these forms of bovine trypsin using chicken ovomucoid, a trypsin inhibitor from egg white, covalently bound to Sepharose. That system is also capable of isolating these enzymes from activated pancreatic juice. Hence one could separate on such a column the trypsin and chymotrypsin eluted by 0.1 M sodium acetate from a soybean trypsin inhibitor–Sepharose column

(Figure 1). The trypsin activity would be fractionated into the α and β forms, while the chymotrypsin activity would appear in the void volume and would thus be unfractionated.

The nature of the carboxypeptidase β isolated gives this work added practical value. The enzyme appears in three forms; two of them, accounting for about 90% of the total enzyme recovered, contain internal splits but are indistinguishable in specific activity from the single-chain form of carboxypeptidase B. The two forms of the enzyme containing the split display only small differences, if any, in amino acid composition from the single-chain form.

The origin of these two chain forms of carboxypeptidase B is not firmly established, but a reasonable explanation is evident. The split forms of the enzyme were isolated from activated pancreatic juice which probably contained more trypsin and chymotrypsin than carboxypeptidase B on a molar basis (Keller *et al.*, 1958). Thus any bond in native carboxypeptidase B which would be susceptible to hydrolysis by these endopeptidases might well be expected to be cleaved. The bonds involved are His_{95}–Met_{96} (carboxypeptidase B I) and Arg_{92}–Glu_{93} (carboxypeptidase B II) using the numbering system for carboxypeptidase A_α (Reeck *et al.*, 1971).

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References

Allan, B. J., Keller, P. J., and Neurath, H. (1964), *Biochemistry 3*, 40.

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 663.

Axén, R., Porath, J., and Ernback, S. (1967), *Nature (London) 214*, 1302.

Barman, T. E., and Koshland, D. E., Jr. (1967), *J. Biol. Chem. 242*, 5771.

Cole, R. D. (1967), *Methods Enzymol. 11*, 315.

Cox, D. J., Bovard, F. C., Bargetzi, J.-P., Walsh, K. A., and Neurath, H. (1964), *Biochemistry 3*, 44.

Cox, D. J., Wintersberger, E., and Neurath, H. (1962), *Biochemistry 1*, 1078.

Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 636.

Davis, B. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1*, 80.

Folk, J. E., and Gladner, J. A. (1959), *Biochim. Biophys. Acta 33*, 570.

Folk, J. E., Piez, K. A., Carroll, W. R., and Gladner, J. A. (1960), *J. Biol. Chem. 235*, 2272.

Hermodson, M. A., Ericsson, L. H., and Walsh, K. A. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 728.

Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 59.

Horbett, T. A. (1970), Ph.D. Thesis, University of Washington.

Hughes, T. R., and Klotz, I. M. (1956), *Methods Biochem. Anal. 3*, 265.

Hummel, B. C. W. (1959), *Can. J. Biochem. Physiol. 37*, 1393.

Keller, P. J., Cohen, E., and Neurath, H. (1958), *J. Biol. Chem. 233*, 344.

Kycia, J. H., Elzinga, M., Alonzo, N., and Hirs, C. H. W. (1968), *Arch. Biochem. Biophys. 123*, 336.

Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Jr., Quiocho, F. A., Ludwig, M. L., Steitz, T. A., Bethge, P., Muirhead, H., and Coppola, J. C. (1968), *Brookhaven Symp. Biol. 21*, 24.

McClure, W. O., Neurath, H., and Walsh, K. A. (1964), *Biochemistry 3*, 1897.

Pétra, P. H., and Neurath, H. (1969), *Biochemistry 8*, 2466.

Pétra, P. H., Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8*, 2762.

Porath, J., Axén, R., and Ernback, S. (1967), *Nature (London) 215*, 1491.

Putnam, F. W., and Neurath, H. (1946), *J. Biol. Chem. 166*, 603.

Reeck, G. R., Walsh, K. A., Hermodson, M. A., and Neurath, H. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1226.

Robinson, N. C., Tye, R. W., Neurath, H., and Walsh, K. A. (1971), *Biochemistry 10*, 2743.

Sanders, T. G. (1970), Ph.D. Thesis, University of Illinois.

Sanger, F., and Tuppy, H. (1951), *Biochem. J. 49*, 463.

Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci. 164*, 66.

Walsh, K. A., Ericsson, L. H., and Neurath, H. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1339.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Whitaker, J. R., Menger, F., and Bender, M. L. (1966), *Biochemistry 5*, 386.

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

Yphantis, D. A. (1964), *Biochemistry 3*, 297.