Purification and Properties of Dipeptidyl Transferase (Cathepsin C)*

Robert M. Metrione, Armando G. Neves,† and Joseph S. Fruton

ABSTRACT: Dipeptidyl transferase (formerly termed cathepsin C) has been extensively purified from beef spleen by means of gel-filtration and ion-exchange chromatography. The product appears to be nearly homogeneous as judged by its sedimentation behavior. A molecular weight of 210,000 has been calculated from sedimentation equilibrium measurements. Upon treatment with *p*-mercuribenzoate or sodium dodecyl sulfate, the protein undergoes dissociation to yield subunits.

The enzyme specificity of the highly purified enzyme toward synthetic substrates is similar to that of cruder preparations. Dipeptidyl transferase exhibits negligible proteinase activity, indicating that it cannot be considered to be a major component of the cathepsin complex of animal tissues.

Extracts of animal tissues such as spleen, liver, or kidney contain a variety of enzymes that cleave peptide bonds (Fruton, 1960). The nomenclature of these enzymes is in an unsatisfactory state, largely because of the traditional use of the term cathepsin to designate proteolytic activity found in animal tissues, and because the purification of most of the cathepsins has been attended by considerable difficulty. The success in defining the specificity of crystalline proteinases such as trypsin by means of synthetic substrates encouraged the hope that the use of such substrates, in place of proteins, would permit the definition of the individual members of the cathepsin complex (Fruton, 1957). By the use of synthetic substrates, evidence was obtained for the existence of at least three separate enzymes (denoted cathepsins A–C), whose specificities at first appeared to be similar to those of pepsin, trypsin, and chymotrypsin, respectively (Tallan *et al.*, 1952). Cathepsin A hydrolyzes benzyloxycarbonyl-L-glutamyl-L-tyrosylglycine (a pepsin substrate) at the glutamyl–tyrosyl linkage near pH 5 and does not require activation by sulfhydryl compounds; its status is unclear, however, partly because of its considerable lability (Lichtenstein and Fruton, 1960). Cathepsin B is a sulfhydryl-dependent enzyme with a specificity similar to that of trypsin, and purified preparations not only readily cleave benzoyl-L-argininamide (or ethyl ester), but also catalyze the conversion of trypsinogen to trypsin near pH 5 (Greenbaum and Fruton, 1957; Greenbaum *et al.*, 1959). Cathepsin C, the subject of the present communication, was found in tissue extracts through the use of a substrate of chymotrypsin, glycyl-L-phenylalaninamide (Gutmann and Fruton, 1948), but subsequent work showed this enzyme to have a much narrower specificity than that of the pancreatic proteinase (Wiggans *et al.*, 1954; Izumiya and Fruton, 1956). These studies indicated that cathepsin C preferentially attacks the CO group of an amide (or ester) bond located two α-amino acid residues from an α-amino (or α-imino) group. A partial purification of cathepsin C was achieved in this laboratory (Tallan *et al.*, 1952; de la Haba *et al.*, 1959), and the kinetics, activation, and inhibition of such enzyme preparations were studied (Fruton and Mycek, 1956).

In addition to the three proteolytic activities mentioned above, a cathepsin D has been described by Press *et al.* (1960) and Lapresle and Webb (1960). Purified preparations of cathepsin D from spleen cleaved hemoglobin and serum albumin at acid pH values, but failed to split synthetic substrates for cathepsins A–C. A similar enzyme preparation, with higher activity toward hemoglobin than cathepsin D, was obtained by Evtikhina *et al.* (1963); it is unclear at present whether their enzyme is identical with that described by Press *et al.* (1960). Furthermore, Lapresle and Webb (1962) have reported the presence, in extracts of bone marrow, of a proteinase different from cathepsin D, which they have termed cathepsin E. In addition to the enzymes designated cathepsins A–E, and other proteinase preparations related to them, a variety of peptidases (aminopeptidases, carboxypeptidases, etc.) have been found in extracts of animal tissues. For a recent discussion of the uncertainties in the classification of some of the aminopeptidases, see Patterson *et al.* (1965).

Our interest in cathepsin C stems largely from the finding that this enzyme is exceptional, among proteolytic enzymes, in the efficiency with which it catalyzes transamidation reactions (Jones *et al.*, 1952; Fruton *et al.*, 1953; Fujii and Fruton, 1958; Würz *et al.*, 1962; Nilsson and Fruton, 1964); *e.g.*, at pH 7.5, the sub-

* From the Department of Biochemistry, Yale University, New Haven, Connecticut. *Received February 4, 1966.* This work was aided by grants from the U. S. Public Health Service (GM-06452) and the National Science Foundation (G-7451).

† Fellow of the Rockefeller Foundation. Present address, Department of Biochemistry, Faculty of Medicine, University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

strate L-alanyl-L-phenylalaninamide is converted in high yield to the hexapeptide amide Ala-Phe-Ala-Phe-Ala-Phe-NH_2 (Nilsson and Fruton, 1964); at pH 5, however, the dipeptide amide is hydrolyzed quantitatively to alanylphenylalanine and ammonia. Other substrates (*e.g.*, glycyl-L-phenylalaninamide, glycyl-L-tryptophanamide) are polymerized at pH 7.5 to octapeptide amides. In the presence of a suitable amine (*e.g.*, L-argininamide), the polymerization is inhibited, and a transamidation product (*e.g.*, glycyl-L-phenylalanyl-L-argininamide) is formed (Jones *et al.*, 1952; Planta *et al.*, 1964). Clearly, the enzyme catalyzes a transfer reaction in which a dipeptidyl group is transferred to an acceptor which may be either an unprotonated amino group or water (Jones *et al.*, 1952; Fruton, 1957). Because of the specificity of the enzyme toward dipeptidyl derivatives, and its ability to catalyze transfer to suitable acceptors, we suggest that the term cathepsin C be replaced by the term dipeptidyl transferase. It may be added that the conclusions previously drawn regarding the specificity of cathepsin C in the catalysis of transfer reactions have been confirmed recently by Planta *et al.* (1964).

For the study of the mechanism of the catalytic action of dipeptidyl transferase as a polymerase (see Würz *et al.*, 1962), highly purified preparations are needed, and since the report of de la Haba *et al.* (1959), we have attempted to develop a reproducible method for the isolation of a homogeneous preparation of this enzyme from beef spleen. The purpose of this communication is to report the present state of our work on this problem, in view of two recent publications on the subject. Planta and Gruber (1964) reported efforts to effect the chromatographic purification of the preparation of de la Haba *et al.*, but a homogeneous material does not appear to have been obtained. Dhar and Bose (1964) reported the crystallization of cathepsin C, starting with the preparation of de la Haba *et al.;* no data are given, however, for the action of their enzyme preparation on synthetic substrates, the cleavage of egg albumin or gelatin having been used as a measure of enzymic activity. In view of the finding in this laboratory, and by Planta and Gruber (1961), that partially purified preparations of cathepsin C do not attack proteins (hemoglobin, serum albumin, egg albumin, insulin, ribonuclease) extensively, further studies on the specificity of the preparation of Dhar and Bose are needed.

Experimental Section

Enzyme Assay. The transamidation assay of de la Haba *et al.* (1959) was modified as follows: Into a 2-ml volumetric flask was pipetted 0.1 ml of 2 M NH_2OH (pH 6.8; prepared fresh from 4 M NH_2OH hydrochloride by adjustment with 4 N NaOH), 0.1 ml 0.125 M β-mercaptoethylamine (pH 6.8; prepared freshly from a stock solution of the hydrochloride by adjustment with 0.1 N NaOH), 0.1 ml of 0.25 M glycyl-L-phenylalaninamide acetate (adjusted to pH 6.8 with 0.1 N NaOH), and 0.1 ml of water. This mixture was kept at 37° for 5 min, and 0.1 ml of the enzyme solution (appropriately diluted) was added at zero time. The reaction was stopped after 10 min (at 37°) by the addition of 0.5 ml of 20% trichloroacetic acid, followed by 0.5 ml of 5% $FeCl_3 \cdot 6H_2O$ in 0.1 N HCl. The volume was brought to 2 ml with water, and the absorbance at 510 mμ was read within 10 min in a Coleman Jr. spectrophotometer (10 × 75 mm cuvets) *vs.* a blank prepared as described above except that the enzyme solution was replaced by 0.1 ml of water. Proportionality between enzyme concentration and absorbance is observed only below an absorbance of 0.25.

One enzyme unit is defined as the amount of dipeptidyl transferase that catalyzes the formation of 1 μmole of hydroxamic acid/min under the above conditions of assay. The specific activity is defined as enzyme units per milligram of protein, as determined by means of the biuret method of Gornall *et al.* (1949). For the calculation of the amount of hydroxamic acid produced, the standard curve obtained with benzoyl-L-alanylhydroxamic acid (Johnston *et al.*, 1950) was used. The enzyme unit defined above is 0.1 of that used by de la Haba *et al.* (1959).

A convenient method for determining whether a fraction contains dipeptidyl transferase activity is a spot-test adaptation of the quantitative assay. The reagents are the same as those described above. On a spot plate add: 1 drop each of substrate, β-mercaptoethylamine, and NH_2OH. After mixing, add 1–2 drops of solution to be tested for activity. Let stand at room temperature for defined period (3–10 min), add 1 drop of 2 N HCl, followed by 1 drop of $FeCl_3$.

The hydrolytic action of purified dipeptidyl transferase on peptide amides and amides at pH 6 was determined by measurement of ammonia liberation, using the diffusion method of Seligson and Seligson (1951). The following substrates have been described previously: glycyl-L-phenylalaninamide acetate and glycyl-L-tyrosinamide acetate (Fruton and Bergmann, 1942), glycyl-L-tryptophanamide *p*-toluenesulfonate (Theodoropoulos and Fruton, 1962). The synthesis of L-histidyl-L-phenylalaninamide diacetate and of L-histidyl-L-tyrosinamide monoacetate will be described in a separate communication. Glycyl-L-phenylalanine *p*-nitroanilide was a gift of Professor J. Rudinger; a modification of the spectrophotometric procedure of Planta and Gruber (1963) was used to follow the cleavage of this substrate. We are indebted to Dr. E. Boedefeld for the data on the hydrolysis of the synthetic substrates.

Purification of Dipeptidyl Transferase. In what follows, we give a typical laboratory-scale preparation from *ca.* 12 lb of fresh beef spleen. The outer membrane was removed from the organs, which were then cut into strips *ca.* 5 cm wide, placed in a plastic pan, and frozen for 8 hr. After being allowed to thaw overnight in the cold room, the tissue was passed twice through a meat grinder, yielding 4667 g of material, to which 9334 ml of water (containing 5.6 g of EDTA) was added. To the stirred mixture, 150 ml of 6 N H_2SO_4 was added to bring the pH to 3.5. The stirring was continued at

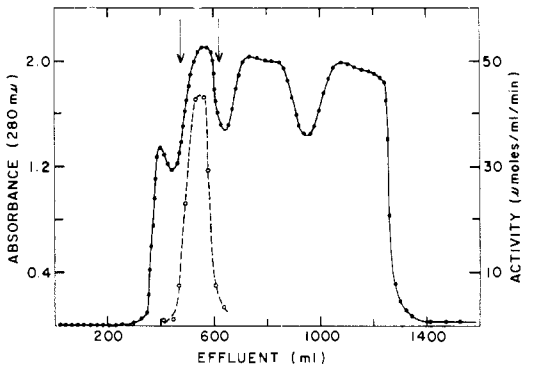

FIGURE 1: Typical elution pattern of heated fraction of beef spleen dipeptidyl transferase on Sephadex G-200 at 20°. Fractions of 11 ml were collected at a flow rate of 60 ml/hr. Solid line, absorbance at 280 mμ; dashed line, enzyme activity. The arrows denote the G-200 fraction.

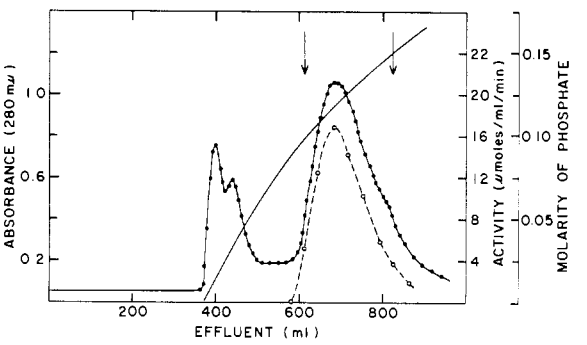

FIGURE 2: Typical elution pattern of G-200 fraction of beef spleen dipeptidyl transferase on DEAE-cellulose at 0°. Fractions of 6 ml were collected at a flow rate of 24 ml/hr. Solid line with points, absorbance at 280 mμ; dashed line, enzyme activity; solid line without points, gradient. The arrows denote the DEAE fraction.

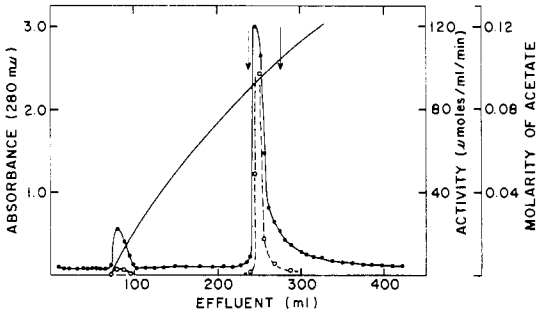

FIGURE 3: Typical elution pattern of DEAE fraction of beef spleen dipeptidyl transferase on CM-cellulose at 0°. Fractions of 6 ml were collected at a flow rate of 18 ml/hr. Solid line with points, absorbance at 280 mμ; dashed line, enzyme activity; solid line without points, gradient. The arrows denote the CM fraction.

room temperature for 1 hr, and the pH was readjusted to 3.5 with 10 ml of 6 N H_2SO_4. The suspension was incubated at 38° for 22 hr (with slow stirring for the first 5 hr), and after the solid material had settled, the supernatant fluid was siphoned off and the residue was centrifuged at 10,000*g*. The combined supernatant fluids are designated acid extract.

To the acid extract, 2960 g of ammonium sulfate (40% of saturation) was added with stirring. After 2 hr, 40 g of acid-washed Hyflo filter cel was added, and the suspension was filtered with suction using large sheets of soft filter paper (Schleicher and Schull, No. 589). To the filtrate (13.4 l.), 2750 g of ammonium sulfate was added with stirring to bring its concentration to 70% of saturation. After the suspension had been kept in the cold room overnight, it was filtered with suction through hardened filter paper (Schleicher and Schull, No. 576). The precipitate was suspended in about 70 ml of water and dialyzed extensively *vs.* 0.155 M (0.9%) NaCl to yield the 40–70 AS fraction.[1]

The 40–70 AS fraction was divided into *ca.* 15-ml portions, heated to 65° for 40 min, and chilled in ice. The resulting precipitate was removed by centrifugation, and the supernatant fluids were pooled to yield the heated fraction.

A portion (50 ml) of the heated fraction was applied to a Sephadex G-200 column (5 × 73 cm) and elution was performed with 0.9% NaCl. This operation may be conducted at room temperature (20–25°) without loss of activity. A typical effluent diagram is shown in Figure 1. The effluent solution obtained at 440–615 ml (see arrows in Figure 1) was taken and concentrated by precipitation with 80% ammonium sulfate (special enzyme grade, Mann Research Laboratories). After 30 min, the precipitate was collected by centrifugation, suspended in 5 ml of water, and dialyzed *vs.* 0.005 M sodium phosphate buffer (pH 6.8) containing 0.002 M β-mercaptoethanol, to yield the G-200 fraction. This operation was repeated with the remainder of the heated fraction.

A column of DEAE-cellulose (Brown Co., Selectacel reagent grade, 0.83 mequiv/g) of dimensions 4 × 35 cm was prepared and equilibrated with 0.005 M sodium phosphate buffer (pH 6.8) containing 0.002 M β-mercaptoethanol. A portion of the G-200 fraction (5.2 ml) was applied to the column, and gradient elution was begun immediately by passing 0.25 M phosphate buffer (pH 6.7) containing 0.002 M β-mercaptoethanol into a mixing chamber containing 250 ml of the 0.005 M buffer. This operation may be conducted at room temperature without loss of activity. A typical effluent diagram is shown in Figure 2. The effluent solution obtained at 612–822 ml (see arrows in Figure 2) was taken and

[1] After the development of the complete purification procedure, we were greatly aided in the accumulation of adequate amounts of dipeptidyl transferase by the New England Enzyme Center, where relatively large quantities of beef spleen (up to 400-lb lots) were processed as far as the 40–70 AS fraction. We wish to acknowledge gratefully the help of Dr. Stanley E. Charm, the Scientific Director of the Center, and his staff.

TABLE I: Purification of Beef Spleen Cathepsin C.

Fraction[a]	Vol (ml)	Total Activity (units)	Total Protein (mg)	Sp Act. (units/mg)
Acid extract	12,180	34,100	120,000	0.28
40–70 AS fraction	194	22,700	12,300	1.85
Heated fraction	178	19,200	6,870	2.79
G-200 fraction	18.5	12,100	666	19.2
DEAE fraction	16.8	6,040	286	21.1
CM fraction	7.2	3,690	142	25.9

[a] For the description of each of these fractions, see Experimental Section.

concentrated by precipitation with 80% ammonium sulfate, followed by dialysis *vs.* 0.005 M sodium acetate buffer (pH 5.0) containing 0.002 M β-mercaptoethanol, to yield the DEAE fraction.

A column of CM-cellulose (Mann Research Laboratories, Mannex-CM, Type 20) of dimensions 2 × 31 cm was prepared and equilibrated with 0.005 M sodium acetate buffer (pH 5.0) containing 0.002 M β-mercaptoethanol. A fraction of the DEAE fraction (4.7 ml) was applied to the column and gradient elution was begun immediately by passing 0.35 M sodium acetate buffer (pH 5.0) containing 0.002 M β-mercaptoethanol into a mixing chamber containing 250 ml of the 0.005 M buffer. This operation may be conducted at room temperature without loss of activity. A typical effluent diagram is shown in Figure 3. The effluent solution obtained at 245–272 ml (see arrows in Figure 3) was taken, concentrated by precipitation with 80% ammonium sulfate, dialyzed *vs.* 0.9% NaCl, and stored at 3°. This fraction is designated CM fraction. Over a period of 9 weeks at 3°, the CM fraction was found to retain its activity in the presence of 0.155 M NaCl, 0.1 M KCl, 0.1 M sucrose, or 0.86 M ammonium sulfate, but was inactivated 26% in 0.15 M sodium acetate buffer, pH 5.0. Storage in water alone at 0 or −15° caused the loss of approximately one-half of the original activity within 5 days.

Determination of Sugar Content. For the determination of hexoses by the anthrone method (Scott and Melvin, 1953), a solution containing 1–2 mg of protein was treated in the manner described by Ota *et al.* (1964). Hexosamine was determined by the Elson–Morgan method as modified by Boas (1953), and sialic acid was determined by the thiobarbituric acid method (Warren, 1959). Paper chromatography of hexoses was performed in the manner described by Rosevear and Smith (1961), using their solvent 1 and color reagent II. Neuraminidase (*Vibrio cholerae*) was obtained from General Biochemicals (500 units/ml; Lot No. 54546) and tested for its action on dipeptidyl transferase at pH 5.4 (0.1 M acetate) and 37°, a neuraminidase concentration of 20 units/ml, and a dipeptidyl transferase concentration of 19 units/ml.

Physical Studies. The ultracentrifuge experiments were performed with a Spinco Model E instrument equipped with a phase plate as a schlieren diaphragm and with a rotor temperature indicating control. For the sedimentation velocity experiments, the conventional 12-mm cell was used. The double-sector-filled Epon cell was used for the sedimentation equilibrium experiments. The partial specific volume was determined by means of density measurements according to Linderstrøm-Lang and Lanz (1938), using the gradient forming device suggested by Riggsby and Rappaport (1965).

Results

Purification. The purification of beef spleen dipeptidyl transferase, as described in the Experimental Section of this communication, departs from several of the previous procedures in the following respects: (1) Whereas most recent investigations (Planta and Gruber, 1961, 1964; Dhar and Bose, 1964) have used for the initial steps in the purification the method of de la Haba *et al.* (1959), we have found it more efficient to use a modified form of the procedure described by Tallan *et al.* (1952). This markedly shortens the time of the operation, and provides material suitable for the subsequent steps. (2) The heat treatment is followed by gel filtration on Sephadex G-200. A typical elution diagram is given in Figure 1, and confirms the observation of Planta and Gruber (1964) that this is a useful step in the purification of the enzyme. (3) In contrast to the negative results reported by Planta and Gruber (1964), we have been able to combine gel filtration and ion-exchange chromatography by successively passing the G-200 fraction through a DEAE-cellulose column (Figure 2) and then a CM-cellulose column (Figure 3). A summary of the procedure developed thus far is given in Table I. In a series of preparations, with varying amounts of starting material, the yield of CM-fraction ranged from 300 to 800 units/kg of fresh beef spleen, with a specific activity ranging from 18 to 28 units/mg protein.

It will be noted that the procedure summarized in Table I achieves an increase in specific activity of about 90-fold, from the acid extract to the CM fraction. This may be compared with the 10-fold increase over the acid extract achieved by the procedure of de la Haba *et al.* (1959). Earlier work had shown (Tallan *et al.*,

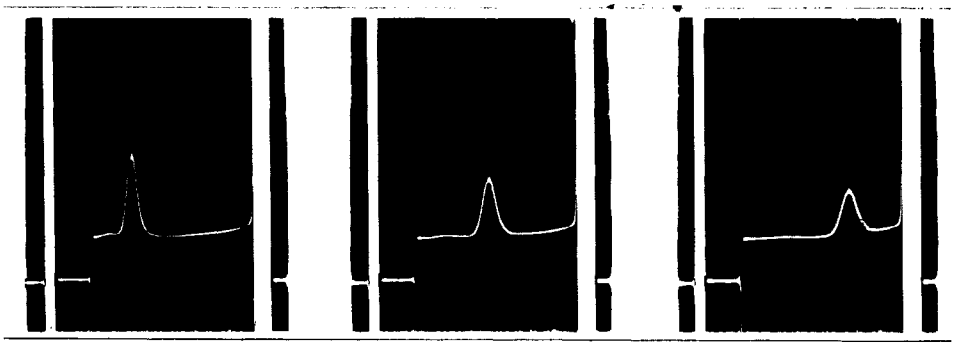

FIGURE 4: Ultracentrifuge pattern of beef spleen dipeptidyl transferase (CM fraction). The solution contained 5.4 mg of protein/ml in 0.1 M sodium acetate buffer, pH 5.4, and 0.002 M β-mercaptoethanol. Photographs, from left to right, were taken at 16, 32, and 48 min after full speed (59,780 rpm) had been attained; temperatures: initial 20.0°; final 20.1°. Phase angle 60°.

1952; de la Haba *et al.*, 1959) that the step from the crude aqueous extract (not shown in Table I) to the acid extract leads to an increase of about 20-fold in specific activity. The over-all purification achieved by the method described in this communication is, therefore, *ca.* 1800-fold.

Sedimentation Behavior. The preparation obtained in this manner appears to be nearly homogeneous upon ultracentrifugation in the pH range 5.3–6.4 ($\gamma/2$ 0.1); a representative pattern for pH 5.4 is shown in Figure 4. The dependence of the sedimentation coefficient on the concentration of protein is given by the equation $s_{20,w} = 9.73(1 - 0.02c)$ S, where c is the concentration of protein (dry weight) in g/100 ml.

The apparent molecular weight of the CM fraction was determined by the application of the calculation procedure of Van Holde and Baldwin (1958) to the results of two sedimentation equilibrium experiments, one of which is shown in Figure 5. The *z*-average molecular weights calculated were 213,000 ± 2000 and 200,000 ± 2000. The partial specific volume of the enzyme was found to be 0.73 ml/g.

Dissociation into Subunits. The relatively small concentration dependence of the sedimentation coefficient of dipeptidyl transferase suggests that it is a compact globular macromolecule (Schachman, 1960). In view of its appreciable size, it appeared likely that the protein represents an aggregate of smaller subunits. A further indication of this possibility was the finding that, upon chromatographic purification, enzyme preparations became cold labile, and frequently lost activity upon storage at −15°. Several enzymes have been shown to be inactivated upon freezing, and it has been found that the loss of activity is accompanied by dissociation (for references, see Scrutton and Utter, 1965).

More definitive indication of the polymeric nature of dipeptidyl transferase was provided by examination of the sedimentation behavior of the CM fraction in the presence of 0.001 M *p*-mercuribenzoate or of 1% sodium dodecyl sulfate. With *p*-mercuribenzoate, a solution of the enzyme (7 mg of protein/ml; 0.1 M sodium acetate buffer, pH 5.4) was found to contain, in addition to unchanged material sedimenting at 9.5 S, a new component having a sedimentation coefficient of 5.5 S. The latter component accounted for about 25% of the total protein. The original sedimentation pattern (one peak at 9.5 S) could be restored by dialysis of the mercuribenzoate-treated enzyme *vs.* 400 volumes of 0.001 M β-mercaptoethanol. When assayed for enzyme activity by the usual hydroxamate method (in the presence of β-mercaptoethylamine), the mercuribenzoate-treated enzyme was found to be fully active both before and after dialysis *vs.* β-mercaptoethanol. It had been observed previously (Fruton and Mycek, 1956) that this reagent does not inhibit the hydrolytic action of dipeptidyl transferase, when the treated enzyme is tested in the presence of the necessary sulfhydryl activator. Further work is needed to establish whether enzyme activity is retained by the 5.5 S component produced by treatment with mercuribenzoate or whether the original 9.5 S component is rapidly reconstituted

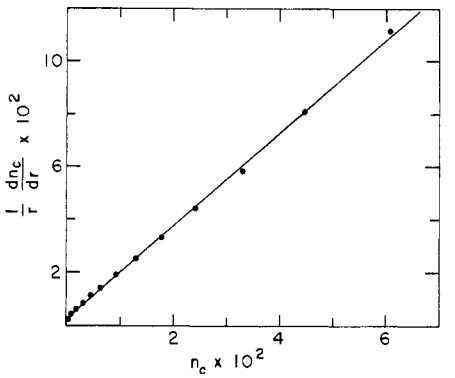

FIGURE 5: Determination of *z*-average molecular weight of beef spleen dipeptidyl transferase (CM fraction). A graph of $(1/r)(dn_c/dr)$ *vs.* n_c (Van Holde and Baldwin, 1958) at 44 hr after attaining a speed of 8225 rpm and at equilibrium. Protein concentration, 3.1 mg/ml in 0.1 M sodium acetate buffer (pH 5.4); temperature, 20.0 ± 0.1°; phase angle, 70°.

by the addition of the sulfhydryl compound used in the enzyme assay. Studies are in progress on the reaction of *p*-mercuribenzoate and of other sulfhydryl reagents (iodoacetate, iodoacetamide, *N*-ethylmaleimide) with purified dipeptidyl transferase. It was found previously (Fruton and Mycek, 1956) that cruder enzyme preparations are inhibited by 0.001 M iodoacetate, and that this effect is not reversed by sulfhydryl compounds.

In the presence of 1% sodium dodecyl sulfate, the enzyme (7.3 mg of protein/ml; 0.1 M sodium acetate buffer, pH 5.4; 0.002 M β-mercaptoethanol) was totally converted to a product sedimenting as a single peak of 1.9 S. The treated enzyme solution was inactive even after dilution and addition of sulfhydryl compound for assay.

Carbohydrate Content. The enzyme preparations were found to contain carbohydrate, and the analytical data for the three fractions obtained by gel filtration and ion-exchange chromatography are given in Table II.

TABLE II: Sugar Content of Enzyme Fractions.[a]

Fraction	Hexose[b]	Hexosamine[c]	Sialic Acid[d]
G-200 fraction	0.059	0.061	0.020
DEAE fraction	0.065	0.064	0.019
CM fraction	0.033	0.032	0.008

[a] Expressed as milligram per milligram of protein in enzyme fraction analyzed. [b] Expressed as glucose. [c] Expressed as glucosamine. [d] Expressed as *N*-acetylneuraminic acid.

In view of the decrease in the sugar content after passage through CM-cellulose, it would appear that at least a portion of the carbohydrate in the crude enzyme preparations is not associated with the enzyme. Further work is needed to establish whether the residual carbohydrate (presumably a mucopolysaccharide) is an integral part of the enzyme. Treatment of the DEAE fraction with neuraminidase (which released all the bound sialic acid) did not cause any decrease in the specific activity of the enzyme preparation.

Absorption Spectrum. The absorption spectrum of dipeptidyl transferase in 0.1 M sodium acetate pH 5.4 shows no unusual features; the absorbance at the maximum (280 mμ) for a solution (1-cm path length) containing 1 mg/ml is 1.54.

Enzymic Specificity. The hydrolytic activity of the CM fraction was tested with several synthetic substrates of dipeptidyl transferase at pH 6, where transamidation is repressed and the hydrolysis is the predominant reaction (Fruton and Mycek, 1956). The data are given in Table III. The specificity and kinetics of highly purified dipeptidyl transferase will be reported in a future communication; it should be noted here, however, that among the new compounds recently synthesized in this laboratory for test as substrates for dipeptidyl transferase, and cleaved by the enzyme, are L-histidyl-L-phenylalaninamide and L-histidyl-L-tyrosinamide. These dipeptide amides may therefore be added to the extensive series examined earlier with partially purified enzyme preparations (Izumiya and Fruton, 1956; Wiggans *et al.*, 1954; Fruton and Mycek, 1956; Würz *et al.*, 1962). Glycyl-L-phenylalanine *p*-nitroanilide (0.001 M) was cleaved by the CM fraction at an initial rate of 1.6%/min in the presence of 0.56 enzyme unit/ml and 0.004 M β-mercaptoethylamine at pH 6.0 (0.1 M citrate) and 25°. Under the conditions given in Table III, the CM fraction did not cause measurable deamidation of L-tyrosinamide or of benzoyl-L-argininamide in 3 hr. The results obtained with the CM fraction are in accord with the conclusions drawn earlier (Fruton, 1957) about the specificity requirements of the enzyme; these conclusions have recently been confirmed and restated by Planta *et al.* (1964). It may be added that the CM fraction readily catalyzes the polymerization of glycyl-L-phenylalaninamide at pH 7.5, under the conditions described previously (Nilsson and Fruton, 1964).

TABLE III: Hydrolytic Action of Dipeptidyl Transferase.[a]

Substrate	NH_3 Liberation (μmoles/ml) 30 min	60 min
Glycyl-L-phenylalaninamide	18.3	31.5
Glycyl-L-tyrosinamide	18.4	34.0
Glycyl-L-tryptophanamide	20.4	36.3
L-Histidyl-L-phenylalaninamide	9.7	17.5
L-Histidyl-L-tyrosinamide	5.5	11.3

[a] Substrate concentration, 0.05 M; concentration of CM fraction, 0.56 unit/ml; β-mercaptoethylamine, 0.004 M; pH 6.0 (0.1 M citrate); temperature, 38°.

Planta and Gruber (1961, 1964) reported that partially purified preparations of beef spleen dipeptidyl transferase showed no proteinase activity toward hemoglobin, serum albumin, fibrinogen, or ribonuclease. We confirm the absence of appreciable proteinase activity in highly purified preparations of the enzyme. The CM fraction was found to contain only traces of proteinase activity toward bovine hemoglobin (1.43% substrate, 0.1 enzyme unit/ml, 0.01 M cysteine, 0.2 M acetate buffer, pH 3.5, 38°) under the assay conditions described by Tallan *et al.* (1952), even when the incubation period was increased from the usual 10 min to 1 hr. The following proteins, when tested at the indicated concentrations at pH 4.0 (other conditions as above), were not cleaved to a measurable extent: bovine serum albumin (0.48%), egg albumin (1%), ribonuclease (0.25%), insulin (0.13%).

Discussion

In the present communication, a method is described for the preparation, from beef spleen, of highly purified dipeptidyl transferase in the form of a polymeric protein having a $s_{20,w}$ value of 9.73 and an apparent molecular weight of about 210,000. Previous ultracentrifuge studies of dipeptidyl transferase were reported by de la Haba *et al.* (1959) with a preparation purified by acetone fractionation and electrophoresis convection. These investigators observed two components in pH 5.4 sodium phosphate buffer ($\gamma/2$ 0.1) with $s_{20,w}$ values of 3.00 and 8.16 S, the enzymic activity being associated with the more rapidly sedimenting component. Planta and Gruber (1964) reported that the preparation obtained by chromatography on a single column containing separate layers of DEAE-Sephadex and DEAE-cellulose also had two components, with sedimentation coefficients of 2.8 and 9.7 S (pH and ionic strength not given). Planta and Gruber (1964) also reported that the material obtained upon passing the preparation of de la Haba *et al.* through Sephadex G-200 gave two peaks in the ultracentrifuge, with sedimentation coefficients of 6.3 and 8.6 S (pH and ionic strength not given). It would appear that the value of 9.7 S, found by Planta and Gruber (1964) for the heavier component in one of their preparations, most closely approximates the $s_{20,w}$ value reported in the present communication. From their sedimentation diagrams, de la Haba *et al.* (1959) calculated an approximate value of 3×10^{-7} cm^2/sec for the diffusion coefficient of the rapidly sedimenting component. By assuming a partial specific volume of 0.75, they calculated a molecular weight of 235,000 ± 50,000. Planta and Gruber (1964) reported a calculated value of 210,000 ± 30,000 for the 9.7 S component, assuming a partial specific volume of 0.75 (the value of the estimated diffusion coefficient was not given).

The recognition that dipeptidyl transferase represents an aggregate of subunits adds it to the increasing number of enzymes that undergo dissociation under suitable conditions of pH or temperature, or upon the addition of detergents, guanidine, urea, or *p*-mercuribenzoate. Among the known enzymes of this class are rabbit muscle aldolase (Deal *et al.*, 1963), *E. coli* alkaline phosphatase (Schlesinger, 1965), and swine heart fumarase (Kanarek *et al.*, 1964). The availability of a homogeneous preparation of the active polymeric form of dipeptidyl transferase permits the determination of the number of subunits through chemical studies (amino acid composition, end groups of peptide chains, titration of sulfhydryl groups, etc.) and by physical measurements. This work is in progress, and will be reported in a future communication.

Of special interest is the possibility that the action of dipeptidyl transferase as a catalyst of the polymerization of dipeptidyl units may be related to the specific association of the subunits. In a previous communication (Würz *et al.*, 1962) it was suggested that dipeptidyl transferase has at least two equivalent active sites, and that the growing peptide chain is not released from the enzyme during chain elongation. This hypothesis may now be modified to suggest that chain elongation occurs by the cooperative action of catalytic and binding sites in separate subunits. Studies now in progress on the binding of peptides to dipeptidyl transferase, as studied by gel filtration (Fairclough and Fruton, 1966), may offer evidence related to this hypothesis.

As suggested previously, the polymerization reaction catalyzed by dipeptidyl transferase may serve as a model system for the study of the mechanism of enzyme-catalyzed polymerization reactions. The finding that this enzyme represents an aggregate of subunits invites renewed speculation whether the action of other polymerases may involve the cooperative action of subunits, as has already been suggested (Lee-Huang and Cavalieri, 1965) for ribonucleic acid (RNA) polymerase and deoxyribonucleic acid (DNA) polymerase.

The intracellular role of dipeptidyl transferase is unknown. Because of the widespread acceptance of the view that "cathepsin" is associated with lysosomes (de Duve, 1963), whose function is considered to be the degradation of cell constituents, it has been natural to suppose that dipeptidyl transferase is localized in these subcellular elements. The recent studies of Shibko and Tappel (1965) with rat kidney lysosomes indicate, however, the absence of activity toward glycyl-L-tyrosinamide, so that kidney dipeptidyl transferase may be localized in subcellular units other than lysosomes, although the presence of an inhibitor (Finkenstaedt, 1957) does not appear to have been ruled out. Clearly, the narrow specificity and absence of proteinase activity in highly purified preparations of the enzyme render it unlikely that dipeptidyl transferase represents a significant proteinase component of the lysosomal cathepsin complex assayed with hemoglobin as a substrate.

Earlier studies (Izumiya and Fruton, 1956) on the specificity of dipeptidyl transferase indicated a preference for an aromatic amino acid residue (L-phenylalanine, L-tyrosine, L-tryptophan) as the donor of the CO group of the sensitive bond, and for a relatively small side chain on the amino-terminal amino acid residue (glycine, L-alanine, L-serine). The replacement of the latter by leucine or lysine (or ε-benzoyllysine) inhibited enzymic attack at the sensitive bond. It is of special interest, therefore, that L-histidyl-L-phenylalaninamide and L-histidyl-L-tyrosinamide are good substrates of dipeptidyl transferase, although they appear to be hydrolyzed somewhat more slowly than the corresponding glycyl compounds.

References

Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.

Deal, W. C., Rutter, W. J., and Van Holde, K. E. (1963), *Biochemistry 2*, 246.

de Duve, C. (1963), *Ciba Found. Symp., Lysosomes*, 20.

de la Haba, G., Cammarata, P. S., and Timasheff, S. N. (1959), *J. Biol. Chem. 234*, 316.

Dhar, S. C., and Bose, S. M. (1964), *Leather Sci. 11*, 309; (1965) *Chem. Abstr. 62*, 2970c.

Evtikhina, Z. F., Orekhovitch, V. N., and Shpikiter, V. O. (1963), *Vopr. Med. Khim. 9*, 626.
Fairclough, G. F., and Fruton, J. S. (1966), *Biochemistry 5*, 673.
Finkenstaedt, J. T. (1957), *Proc. Soc. Exptl. Biol. 95*, 302.
Fruton, J. S. (1957), *Harvey Lectures 51*, 64.
Fruton, J. S. (1960), *Enzymes 4*, 233.
Fruton, J. S., and Bergmann, M. (1942), *J. Biol. Chem. 145*, 253.
Fruton, J. S., Hearn, W. R., Ingram, V. M., Wiggans, D. S., and Winitz, M. (1953), *J. Biol. Chem. 204*, 891.
Fruton, J. S., and Mycek, M. J. (1956), *Arch. Biochem. Biophys. 65*, 11.
Fujii, S., and Fruton, J. S. (1958), *J. Biol. Chem. 230*, 1.
Gornall, A. G., Boardwill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.
Greenbaum, L. M., and Fruton, J. S. (1957), *J. Biol. Chem. 226*, 173.
Greenbaum, L. M., Hirshkowitz, A., and Shoichet, I. (1959), *J. Biol. Chem. 234*, 2885.
Gutmann, H. R., and Fruton, J. S. (1948), *J. Biol. Chem. 174*, 851.
Izumiya, N., and Fruton, J. S. (1956), *J. Biol. Chem. 218*, 59.
Johnston, R. B., Mycek, M. J., and Fruton, J. S. (1950), *J. Biol. Chem. 185*, 629.
Jones, M. E., Hearn, W. R., Fried, M., and Fruton, J. S. (1952), *J. Biol. Chem. 195*, 645.
Kanarek, L., Marler, E., Bradshaw, R. A., Fellows, R. E., and Hill, R. L. (1964), *J. Biol. Chem. 239*, 4207.
Lapresle, C., and Webb, T. (1960), *Biochem. J. 76*, 538.
Lapresle, C., and Webb, T. (1962), *Biochem. J. 84*, 455.
Lee-Huang, S., and Cavalieri, L. F. (1965), *Science 148*, 1474.
Lichtenstein, N., and Fruton, J. S. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 787.
Linderstrøm-Lang, K., and Lanz, H. (1938), *Compt. Rend. Trav. Lab. Carlsberg 21*, 315.
Nilsson, K. K., and Fruton, J. S. (1964), *Biochemistry 3*, 1220.
Ota, S., Moore, S., and Stein, W. H. (1964), *Biochemistry 3*, 180.
Patterson, E. K., Hsiao, S., Keppel, A., and Sorof, S. (1965), *J. Biol. Chem. 240*, 710.
Planta, R. J., Gorter, J., and Gruber, M. (1964), *Biochim. Biophys. Acta 89*, 511.
Planta, R. J., and Gruber, M. (1961), *Biochim. Biophys. Acta 53*, 443.
Planta, R. J., and Gruber, M. (1963), *Anal. Biochem. 5*, 360.
Planta, R. J., and Gruber, M. (1964), *Biochim. Biophys. Acta 89*, 503.
Press, E. M., Porter, R. R., and Cebra, J. (1960), *Biochem. J. 74*, 501.
Riggsby, W. S., and Rappaport, H. P. (1965), *J. Biol. Chem. 240*, 87.
Rosevear, J. W., and Smith, E. L. (1961), *J. Biol. Chem. 236*, 425.
Schachman, H. K. (1960), *Brookhaven Symp. Biol. 13* (*BNL 608* (*C 22*)), 49.
Schlesinger, M. J. (1965), *J. Biol. Chem. 240*, 4293.
Scott, T. A., and Melvin, E. H. (1953), *Anal. Chem. 25*, 1656.
Scrutton, M. C., and Utter, M. F. (1965), *J. Biol. Chem. 240*, 1.
Seligson, D., and Seligson, H. (1951), *J. Lab. Clin. Med. 38*, 324.
Shibko, S., and Tappel, A. L. (1965), *Biochem. J. 95*, 731.
Tallan, H. H., Jones, M. E., and Fruton, J. S. (1952), *J. Biol. Chem. 194*, 793.
Theodoropoulos, D. M., and Fruton, J. S. (1962), *Biochemistry 1*, 933.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Wiggans, D. S., Winitz, M., and Fruton, J. S. (1954), *Yale J. Biol. Med. 27*, 11.
Würz, H., Tanaka, A., and Fruton, J. S. (1962), *Biochemistry 1*, 19.