Peptide Hydrolases in Mammalian Connective Tissue. II. Leucine Aminopeptidase. Purification and Evidence for Subunit Structure*

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ABSTRACT: A scheme has been devised using DEAE chromatography, benzalkonium chloride, acetone, and ammonium sulfate to purify leucine aminopeptidase from mammalian connective tissue (bovine dental pulp). The connective tissue enzyme is a protease as shown by its action on denatured α-chymotrypsin. Manganous ion dependence and inhibition by EDTA are common features with the kidney enzyme, although the connective tissue enzyme appears not to be activated by magnesium.

The enzyme occurs in five electrophoretically different forms (isozymes) which all display identical enzymatic activity. Amino acid analysis and peptide maps prepared from the isozymes as well as disc electrophoresis of the denatured enzyme show that two kinds of subunits may exist. The molecular weight is estimated to be 400,000, that of the smallest functional unit 100,000. Nonfunctional chains as small as 25,000 molecular weight may be present. The hog kidney enzyme in contrast appears to consist of one kind of subunit and shows a less complex picture on disc electrophoresis.

Leucine aminopeptidase was discovered as a constituent of hog intestine (Linderstrøm-Lang, 1929) and extensively purified from hog kidney by Smith et al. (1941, 1944, 1955) and Spackman et al. (1955). A new procedure to purify the hog kidney enzyme has since been reported by Hanson and Hütter (1966) who also crystallized aminopeptidase from bovine lens proteins (Hanson et al., 1965).

It now appears that leucine aminopeptidase activity is not only ubiquitous in animal tissues but that two kinds of the enzyme exist in different cell compartments. In addition to the "classical" leucine aminopeptidase a particulate enzyme has been isolated from the microsomal fractions of kidney cells (Hanson et al., 1967) and rat liver (Hopsu-Havu and Sarimo, 1967), which is not metal dependent. Whether arylpeptidases and particulate aminopeptidases are the same enzymes and whether they attack protein substrates are not yet clear. The distinction between the "supernatant" aminopeptidase and the arylpeptidase is emphasized by the work of Patterson et al. (1963).

Similarly, it is not clear whether all the classical leucine aminopeptidases (here defined as a metal-dependent exoprotease which also splits leucinamide or small peptides with a free N-terminal amino group) within the tissues of an organism are alike. This is attributable to the fact that detailed information is not available about the purity of existing preparations and the molecular properties of the enzyme protein. Smith and Spackman (1955) and Hanson et al. (1967) have suggested that the molecule (300,000 or 325,000 molecular weight) is too large to be a single-chain protein.

In this paper we report the purification of a classical leucine aminopeptidase from bovine connective tissue (dental pulp) and evidence for an isozyme distribution generated by two nonidentical subunits. Typical features of the enzyme are compared with properties of hog kidney leucine aminopeptidase.

Materials and Methods

For materials and methods used for column chromatography, and thin-layer chromatography, tissue preparation, the measurement of activity, and the definition of the proteolytic coefficient, C_0, see the preceding paper (Schwabe, 1969). Analytical disc electrophoresis was performed according to the methods of Ornstein and Davis (1964), Clarke (1964), and Schwabe (1966). For preparative disc electrophoresis, a vertical gel apparatus (EC Apparatus Corp.) was used without modification. However, the buffers were not circulated. The 7% gel was formed in the running chamber with the wide-slot former in place. Exposed gel surfaces were layered with water. The run was performed with the chamber in vertical position, cooled by tap water. Peptide maps were prepared as described by Katz et al. (1959). Peptides were purchased from Mann Biochemicals and Cyclo Biochemicals, trypsin and hog kidney aminopeptidase from Schwarz Biochemicals. Benzalkonium chloride (Zephiran, 17% aqueous solution) was obtained from a local drug store.

Experimental Section and Results

DEAE-chromatography. Small DEAE-cellulose columns have been used to separate the connective tissue peptidases into three groups (acid cathepsins, aminotripeptidase, and leucine aminopeptidase). Since we are interested in all three groups of enzymes and since previously employed methods would denature one or other group during isolation we tried to scale up the DEAE procedure for preparative work. DEAE type 70 (Schleicher & Schüll) was prepared for column chromatography as described in the preceding paper (Schwabe, 1969). A jacketed column (acrylic, 9 × 100 cm) was packed to a height of 80 cm with DEAE-cellulose (about 500 g dry weight of cellulose powder) in 0.05 M Tris buffer pH 8.0, 10^{-2} M in sucrose. An inlet piston containing a sintered disc was lowered to the surface of the cellulose and secured by a compressed "O" ring. About 50 g of lyophilized connective tissue extract was dissolved in 2 l. of distilled water and permitted to flow onto the column at a rate of 500 ml/hr. A nearly linear gradient of increasing ionic strength was used for elution. The basic buffer (0.05 M Tris, pH 8.0, 10^{-2} M in sucrose and 10^{-3} M in $MgCl_2$) was used as starting elutent. The gradient was produced as follows. Vessel 1 (gallon jug) contained 3.8 l. of starting buffer, vessel 2 (gallon jug), 3.8 l. of buffer, 0.2 M in NaCl, and vessel 3 (Carboy), 6 l. of buffer, 0.5 M in NaCl. The first two vessels (stirred magnetically) were set up to keep a constant volume. The elution rate of 200 ml/hr could be attained by gravity flow of buffers. Fractions (25 ml) were collected in a refrigerated Büchler fraction collector. The effluent was monitored for protein (280 mµ) with a Gilson ultraviolet monitor coupled to a Textar recorder. Activities were detected as described in the preceding paper (Schwabe, 1969). Two fractions were collected corresponding to DEAE I and DEAE II (see Figure 1). More than 90% of the total cathepsin activity was not adsorbed under these conditions and emerged first from the column. Secondly the amino tripeptidase appeared at an ionic strength of ~0.2, overlapping the first fraction and the leucine aminopeptidase which eluted in fraction II (μ = ~0.4). All fractions were concentrated by lyophilization.

Benzalkonium Chloride Precipitation. This quaternary ammonium compound has been known for a number of years for its bacteriostatic action. While early attempts to use this agent for protein precipitation were not very successful, we have found that good purification of the connective tissue leucine aminopeptidase could be achieved with Zephiran. In a typical experiment 10 g of lyophilized DEAE fraction II was redissolved in 500 ml of distilled water. A 1.7% solution of Zephiran in 0.1 M Tris buffer at pH 8.0 (10^{-3} M $MnCl_2$) was added to the enzyme extract in 20-ml increments (Figure 2). The successive precipitates were collected by centrifugation, resuspended, and stirred at 4° overnight in 125 ml of 0.1 M Tris buffer (pH 8.0), 10^{-2} M in $MgCl_2$, 10^{-3} M in $MnCl_2$, and 0.1 M in NaCl. Insoluble matter was removed by low-speed centrifugation. The supernatant of fraction III usually contained 70–80% of the original activity in a sevenfold-purified form.
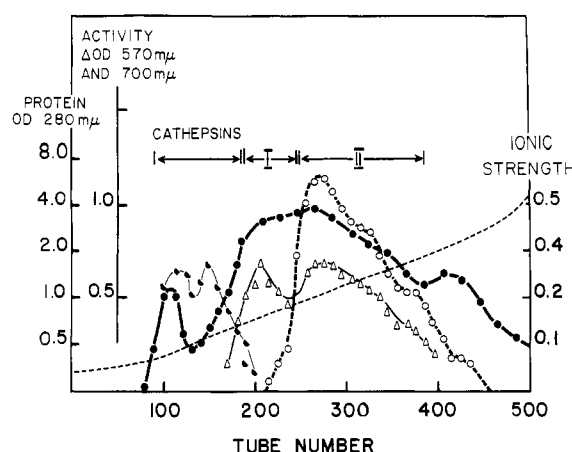

FIGURE 1: The effluent record of a fibroblast extract chromatographed on a preparative DEAE column (9 × 80 cm). The ionic strength gradient indicated in the figure was produced by the addition of various amounts of NaCl to the basic buffer (see text for detail). Cold water (4°) was circulated through the column jacket continuously. Fractions were collected as indicated in the figure. The cathepsins and fraction I (aminotripeptidase) were stored. Fraction II containing the LAP was further purified. (●—●) Protein, (◀—◀) cathepsin activity, (△—△) Leu-Gly-Gly hydrolysis, and (○---○) Leu-Leu hydrolysis. Of the two substrates used to detect peptidases, the aminotripeptidase hydrolyzes Leu-Gly-Gly only, while leucine aminopeptidase hydrolyzes both substrates.

Acetone Fractionation. The enzyme solution (125 ml) obtained from the benzalkonium precipitation procedure (fraction III) was cooled to 0° and cold acetone added in relatively large increments. The precipitate obtained (between 30 and 40% acetone final concentration) was immediately centrifuged at 4000 rpm and redissolved in 10 ml of 0.05 M Tris buffer (pH 8.0), 10^{-3} M in $MgCl_2$. Aliquots of the acetone fractions were lyophilized to remove solvent (which interferes at 280 mµ) to quantify the procedure. The acetone fraction obtained (between 20 and 30% concentration) often contained large amounts of insoluble material from which some aminopeptidase could be reextracted.

Ammonium Sulfate Precipitation. In order to remove most of the residual solvent the acetone fraction was diluted to 300 ml with 0.1 M Tris buffer (pH 8.0) and reconcentrated to 100 ml on an Amicon UM I filter membrane. This solution, containing 1.2 mg of protein/ml, was slowly stirred in an ice bath and 50 ml of saturated ammonium sulfate, containing 1 g of free Tris base/l., was added. No precipitate formed at this point. Solid ammonium sulfate was then added to this solution in 20-g increments. This procedure obviated the foaming usually observed if only solid ammonium sulfate is used. The bulk of the leucine aminopeptidase (70–80%) precipitated from 41 to 58% saturated ammonium sulfate solution, giving rise to a further four- to fivefold purification.

During acetone and ammonium sulfate precipitation all protein and nearly all activity was recovered. Table I lists only the main fractions used in subsequent steps. It is of interest in this context that earlier attempts to

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purify a crude connective tissue extract only with ammonium sulfate, under identical conditions, effected a 70–80% loss of activity.

Agarose Chromatography. Further purification was accomplished through chromatography on a 3 × 150
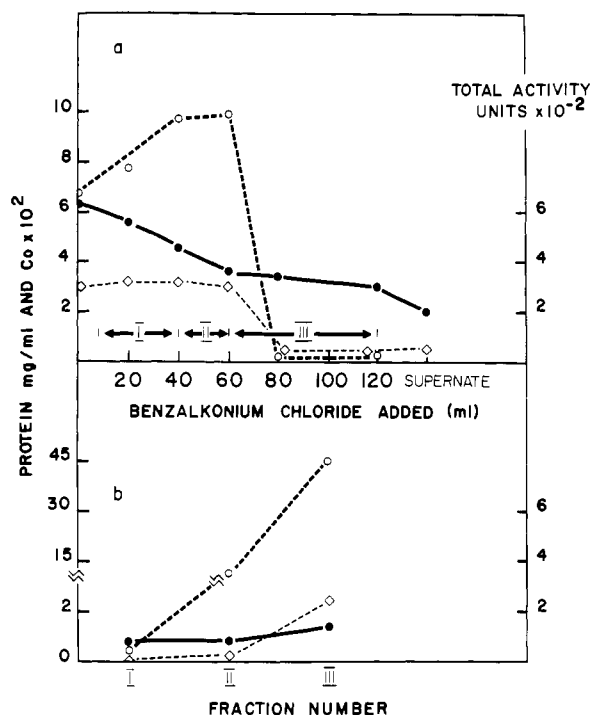

FIGURE 2: Tissue studies. (a) The precipitation of connective tissue leucine aminopeptidase with benzalkonium chloride. Assays were performed on the supernate after each addition of 20 ml of 1.7% benzalkonium in 0.1 M Tris buffer (pH 8.0), followed by low-speed centrifugation. Pellets were combined as indicated in part a by roman numerals. (b) The pellets were resuspended in 125 ml of 10^{-1} M Tris (pH 8.0), 10^{-1} M in NaCl, 10^{-2} M in MgCl, and 10^{-3} M in MnCl$_2$. Activities obtained with the supernate of each pellet (Leu-Leu as substrate) were plotted against pellet number. Pellets were obtained at the following benzalkonium concentrations: I = 0.14%, II = 0.21%, and III = 0.40%. Units: Enzyme amount causing a ΔOD_{570} of 1.0/min under assay conditions described in Methods of the preceding paper (Schwabe, 1969). (○---○) Proteolytic coefficient, C_0 (●—●) total protein; (◊---◊) total activity.

cm Agarose (Bio-Rad) column, the lower two-thirds of which contained Agarose 1.5 (exclusion limit up to 1.5 × 10^6 g/mole) and the upper third contained Agarose 0.5 with an exclusion limit of 5 × 10^5. The enzyme (5 ml) was layered onto the column in 10% sucrose and eluted with 0.025 M Tris buffer at pH 8.0, 10^{-3} M in MgCl$_2$. In Figure 3 it may be seen that the protein peak, although broad, corresponds exactly to the activity obtained. Since single proteins do usually elute in a relatively sharp band from this column it may be concluded that the curve obtained here is due to leucine aminopeptidase units of different size. The range extends from about 400,000 to 100,000 molecular weight, conceivably due to an equilibrium existing between a tetramer and a monomer (mol wt ~100,000).
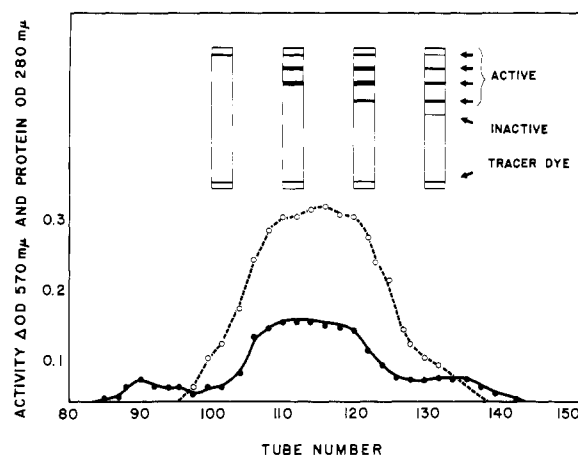

FIGURE 3: Elution record of purified connective tissue leucine aminopeptidase from a column of Agarose (3 × 150 cm). (○---○) Activity (Leu-Leu) and (●—●) protein (280 mμ). The lower 80 cm of the column bed was packed with Agarose 1.5 (1.5 × 10^{-6} exclusion limit) and the upper 40 cm with Agarose 0.5 (5 × 10^5 exclusion limit). The buffer (0.025 M Tris, pH 8.0, 10^{-3} M in MgCl$_2$) was pumped onto the column by a Büchler micropump at a flow rate of 0.3 ml/min. About 3-ml fractions were collected in a refrigerated fraction collector. Inserts depicting disc electrophoresis tubes are redrawn to match relative intensities as closely as possible. The material in the gel tubes was taken from the corresponding portions of the column effluent.

TABLE I: The Purification of Connective Tissue Leucine Aminopeptidase.

Procedure	Vol. (ml)	Protein (mg/ml) OD 280 mμ	Total Protein OD 280 mμ	Sp Act. $C_0 \times 10^2$	Total Act. (units)	Yield (%)	Purity Integers
First extract[a]	1,000	30.0	30,000	1.0	400		1
DEAE fraction	500	6.4	3,200	6.8	300	75	7
Benzalkonium chloride	125	0.6	90	45.0	220	55	45
Acetone (30–40%)	10	10.0	100[b]	80.0	130	32	80
Ammonium sulfate (41–58% saturated)	10	2.7	27	350.0	100	25	350
Agarose column	50	0.25	12.5	600.0[c]	100	25	600

[a] Undialyzed. [b] Probably too high due to residual acetone tightly bound to the protein. [c] An average of several forms of the enzyme possessing different specific activities.

The insert in Figure 3 depicts the results of disc gel electrophoresis of the column fractions below them. Enzyme fractions eluting in the distal part of the column effluent travel further on the 7% acrylamide gel, a fact commensurate with smaller molecule size. Size however cannot be the complete explanation for this occurrence since the equilibrium should immediately restore the larger components.

Disc Electrophoresis. These experiments were performed with a 7% running gel without the use of either a spacer or sample gel. The gel tubes were first placed in the electrophoresis apparatus (Metaloglass, Inc.), the glycine buffer system (Ornstein and Davis, 1964) (including tracer dye) was added, and the electrodes were installed. Last the sample in sucrose was layered directly on the running gel. A temperature of ∼0° was main-

tained by placing ice cubes in the buffer chamber. The upper chamber was lowered to nearly completely submerge the running gels. Triplicate analyses, each of 0.05 mg of purified connective tissue leucine aminopeptidase, were run. The tracer dye was allowed to travel about 5 cm. Half of each gel was then stained with Amido Black and destained in an apparatus previously described (Schwabe, 1966). Essentially five protein bands were obtained. The unstained half was lined up with the stained half and sections (0–4) corresponding to the stained bands were cut out (Figure 4). The gel sections were transferred to 1-ml beakers and 100 μl of Tris buffer at pH 8.0, 10^{-3} M in $MnCl_2$, was added followed by 50 μl of Leu-Leu (5×10^{-3} M). The samples were incubated at 40° for 2 hr after which 5-μl samples from each beaker were spotted on a thin-layer

FIGURE 4: The correlation between bands and activity of connective tissue leucine aminopeptidase isozymes. A gel was sectioned longitudinally and one-half was stained with Amido Black. Corresponding unstained sections were assayed for activity (Leu-Leu as substrate) as described under Disc Electrophoresis. A Leu-Leu control was included (C).
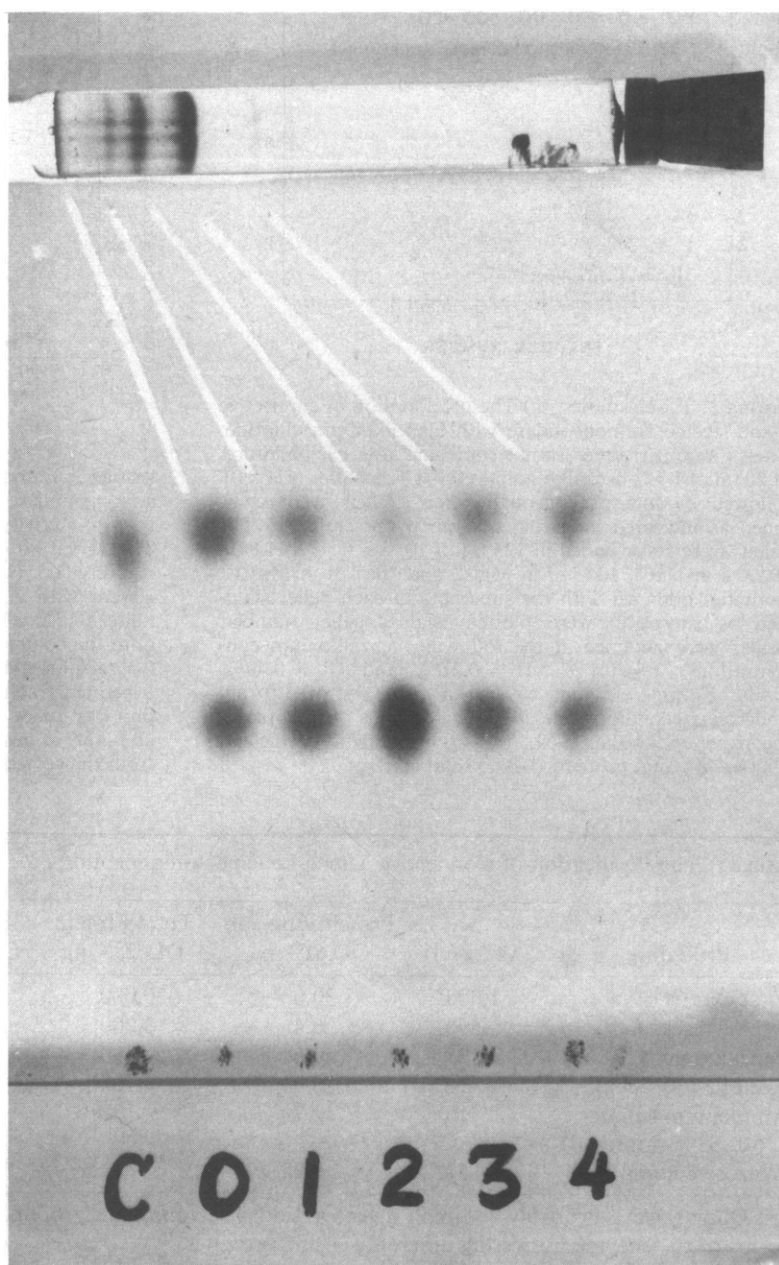
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plate. In Figure 4 the stained longitudinal gel is shown in relation to the spots on the thin-layer plate. The top row of spots result from residual substrate (see Leu-Leu control spot c). The lower row is leucine. Band 2 is most active, most of the Leu-Leu being converted into Leu. All five bands show activity.

Isozymes of leucine aminopeptidase could be generated by size (monomers–dimers–tetramers), or by charge differences (through subunits of differing amino acid composition), or both. From the Agarose column (Figure 3) it is clear that size is at least in part responsible for the creation of the species of leucine aminopeptidase seen on the gel. Importantly, only enzymatically active (leucine aminopeptidase) bands are present. Further evidence for homogeneity of the leucine aminopeptidase is given by the fact that our preparation has no

activity toward denatured papain (endopeptidase contamination free) and peptides with glycine N terminals. The latter activity is strong in commercial hog kidney leucine aminopeptidase.

The Dissociation of Connective Tissue Leucine Aminopeptidase Isozymes by Disc Electrophoresis. If one subunit generates the five leucine aminopeptidase isozymes, all forms should be reproduced from each band. If, however, different subunits are involved this does not necessarily occur. The following experiment was designed to examine the pattern of activity obtained after each isozyme had been subjected to a second disc electrophoresis. The diagram (Figure 5) might aid the understanding of the following description of experimental details. Two samples of the leucine aminopeptidase (0.2 mg/tube) were electrophoresed as described
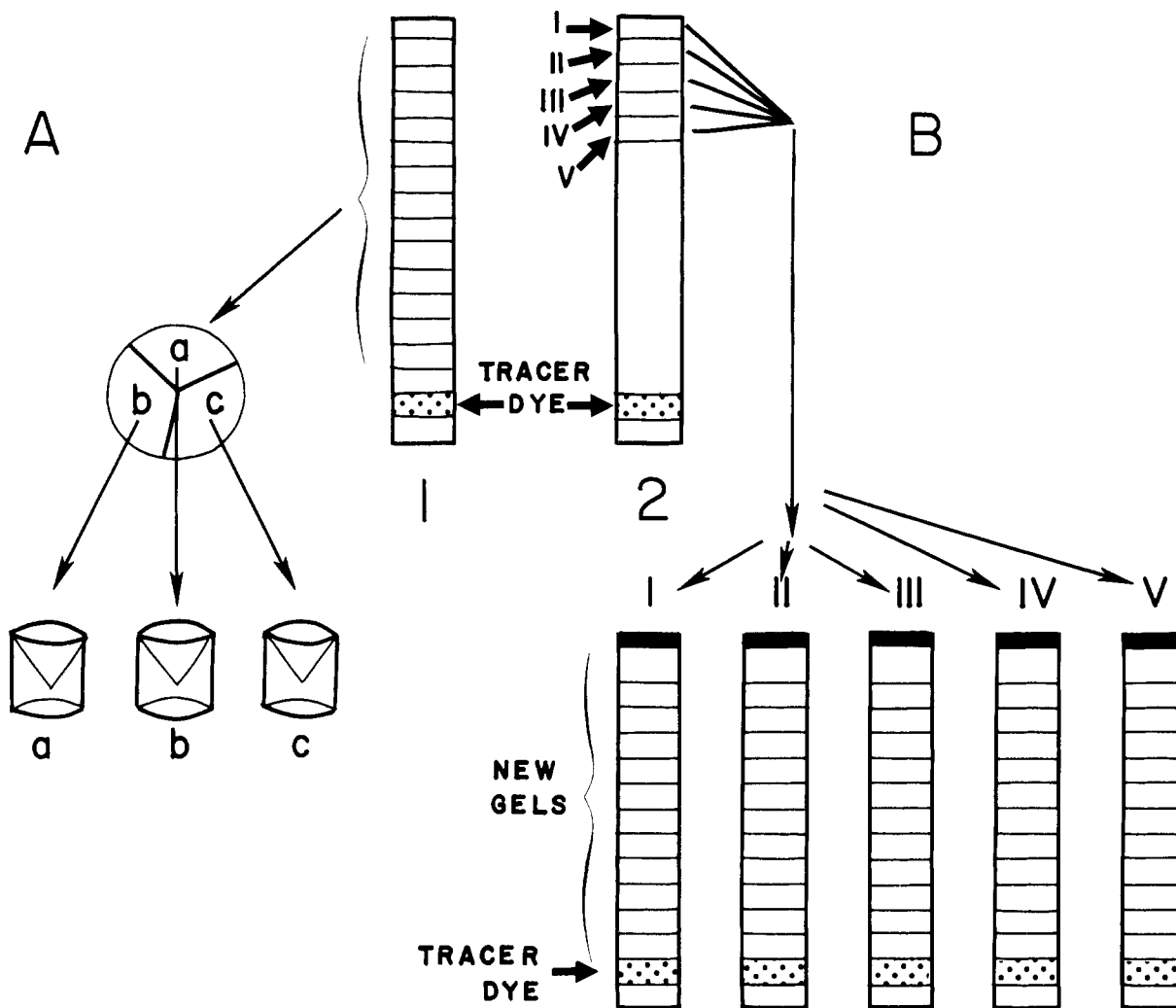

FIGURE 5: The dissociation of isozymes by disc electrophoresis. The experiment is divided into parts A and B as indicated in the figure. Two identical gels (1 and 2) were run to separate the leucine aminopeptidase isozyme. Part A depicts the detection procedure of active isozymes. The gel (1) was sliced into 14, 3-mm thick, sections. Each section was divided into three parts (a, b, c) and transferred to three series of beakers: 14 a pieces were assayed with Leu-Leu, 14 b pieces were assayed with Lys-Leu, and 14 c pieces were assayed with $LeuNH_2$ as substrates. Samples (5 μl) were spotted on thin-layer plates after incubation for 5 hr at 40° (a–c). The first five slices, containing the isozymes, were cut from the second gel (2) and inserted into new glass tubes (I–V). The tubes were inverted and new gels polymerized onto the slices. The tubes were then reelectrophoresed and sliced into 12 sections. The sections were incubated at 40° ($LeuNH_2$ as substrate) and 5 μl was withdrawn from the supernatant of each slice and spotted on thin-layer plates. The developed plates are shown in Figure 6.

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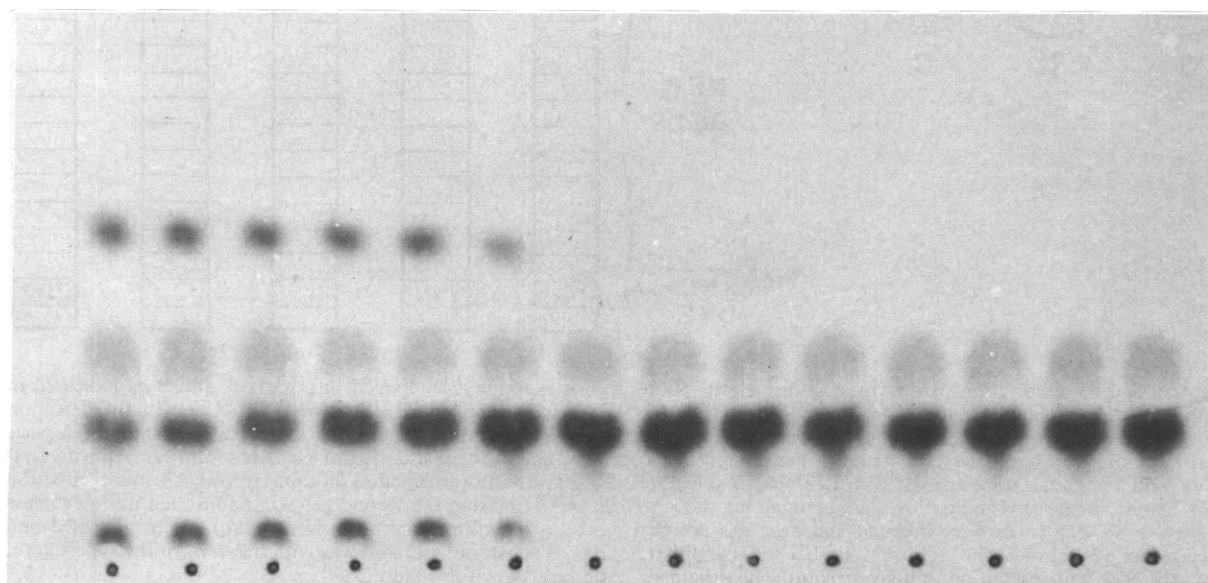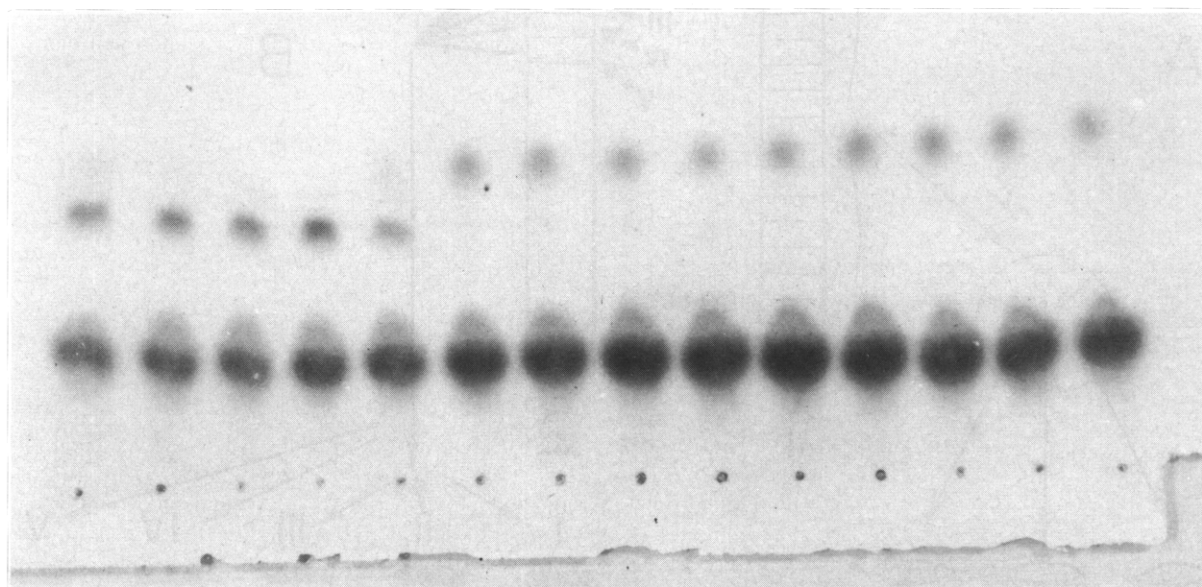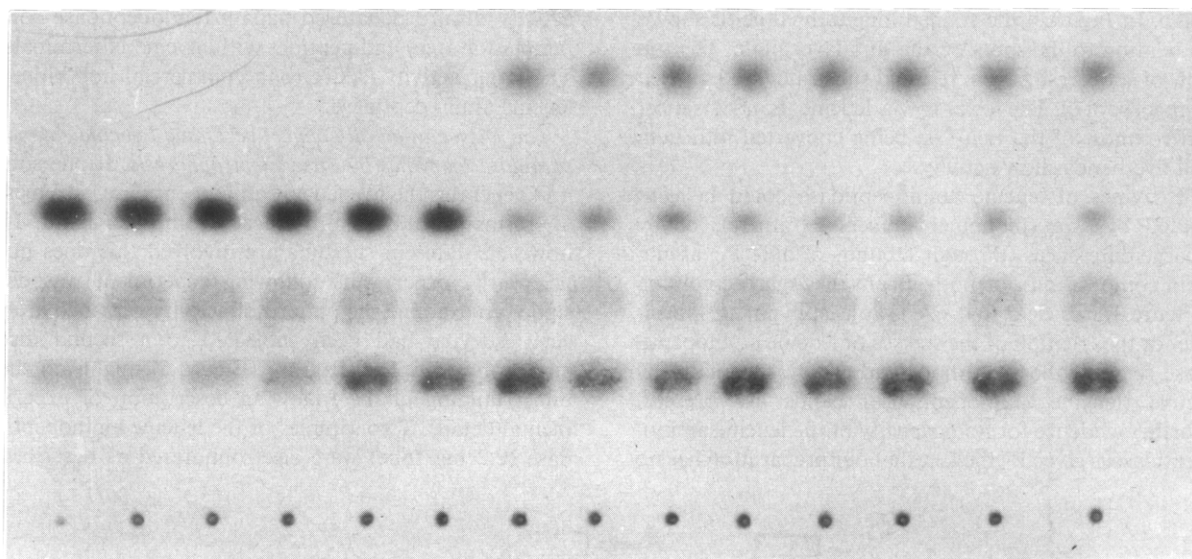
in the previous section. The tracing dye (bromophenol blue) was permitted to travel exactly equal distances on each gel. The cathode portion of the gel, known to contain the isozymes, was cut into 14 (3 mm thick) slices by means of a gel cutter. Each slice was divided into three sections and each section assayed with a different substrate as described under Disc Electrophoresis. Figure 6 shows the thin-layer assays for leucinamide, Leu-Leu, and Lys-Leu. All three substrates were hydrolyzed by the first five fractions (I–V). It should be noted here that the cuts were made without the aid of a stained gel, merely taking equal fractions starting at the top (cathode) side of the gel. Therefore the five fractions do not correspond to five isozymes. The remaining identical set of slices from the second gel was meanwhile transferred to new electrophoresis tubes. Acrylamide was then poured onto these sections and permitted to polymerize. The tubes were then inserted into the disc electrophoresis apparatus (gel slice at the cathode) and the marker dye permitted to run approximately the same distance as on the original gel. Again the 3-mm slices were uniformly cut and assayed with LeuNH$_2$. The thin-layer plates depicted in Figure 7 (1–5) represent reruns of gel sections equivalent to the first five slices of Figure 6a (counting from left to right). The first slice (cathode of the gel) in Figure 6a gave rise to the activity pattern observed in Figure 7 (1), the second slice (Figure 6a) to the activity shown on the second plate (Figure 7 (2)), etc. Every section (except 1) has dissociated into faster and slower components indicating that the isozymes might be composed of smaller units with a different net charge. Comparing Figure 6a with all the reruns in Figure 7 it becomes clear that a simple difference in aggregate size cannot account for this phenomenon.

If the same experiment is repeated and the gels obtained from the reelectrophoresis of each band are stained for protein, a pattern very similar to that obtained by the much more sensitive assay method appears (Figure 8). Still, the two experiments are in close agreement. The dissociation constant of the units must be comparatively large to permit their separation under the influence of an electric field. No factors influencing the distribution of the various isozymes have been found other than aging, which increases the relative amount of the most anionic species.

The Dissociation of Connective Tissue Leucine Aminopeptidase Isozymes by Exclusion Chromatography. If an equilibrium exists between monomer and polymer of the connective tissue leucine aminopeptidase and if a substantial amount of monomer exists at all times, exclusion chromatography should reveal different size fractions of the enzyme. Any particular fraction of active material collected from this column should again give rise to the original size distribution of active units.
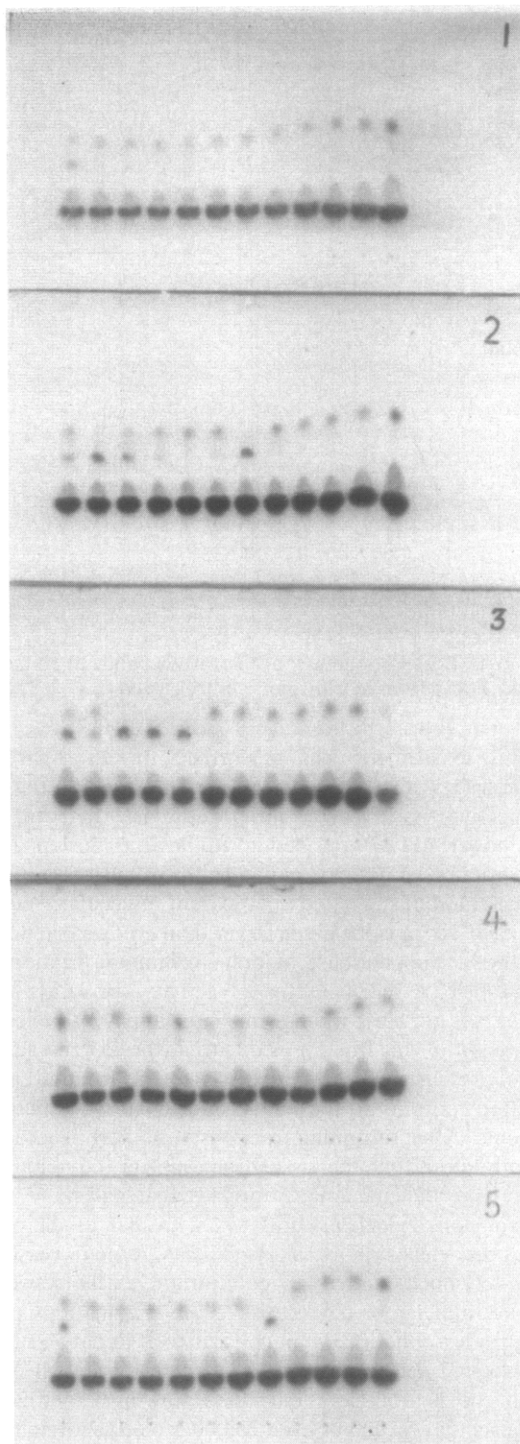

FIGURE 7: Reelectrophoresis of five fractions obtained from a disc gel as depicted in Figure 5. Redistribution of activity into fractions other than the parent fraction from the first gel are seen (see text for detail).

FIGURE 6 (Opposite): Activity of connective tissue leucine aminopeptidase toward (a) LeuNH$_2$, (b) Lys-Leu, and (c) Leu-Leu. LeuNH$_2$, travels further than leucine in phenol. The first five and a trace of the sixth spot indicate production of leucine in part a. (b) The Lys-Leu is hidden under the glycine spot from the disc electrophoresis buffer (present on all plates) but leucine (top line) and lysine (close to the origin) reveal six active spots. (c)The Leu-Leu is the top line of spots and the leucine is clearly seen again in the first six positions. Other leucine spots are contaminants of the substrate. See text for conditions of assay.
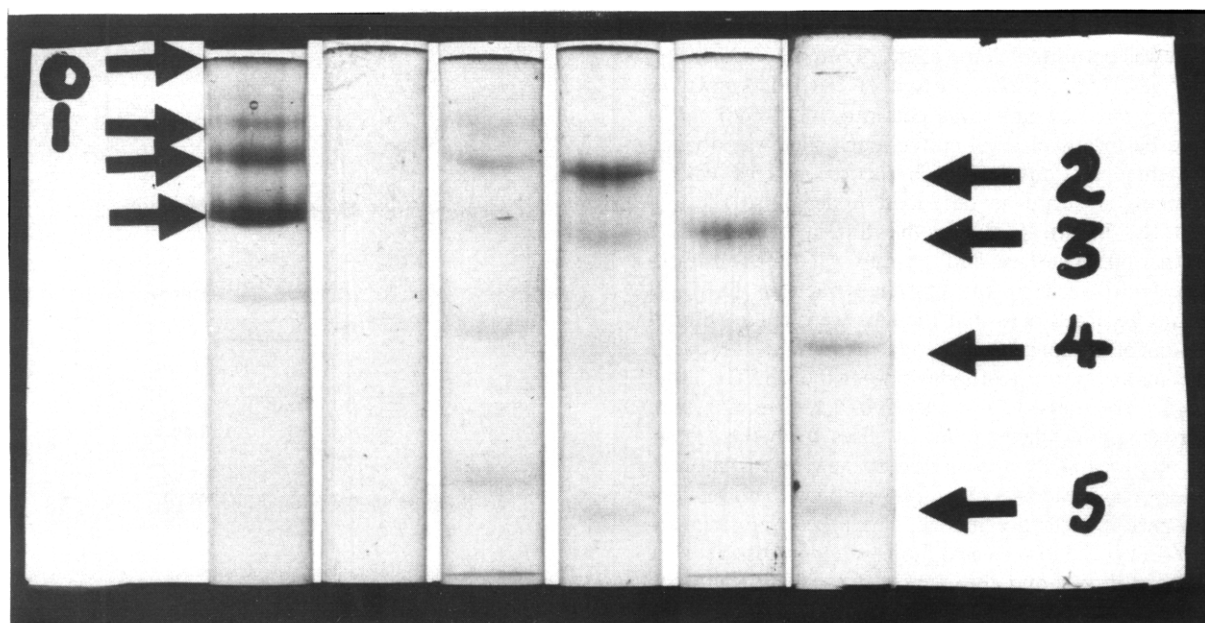
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FIGURE 8: Reelectrophoresis of the varous bands of connective tissue leucine aminopeptidase followed by staining with Amido Black. Tubes from left to right: control, band 0, 1, 2, 3, and 4.

This experiment was performed on an Agarose column (1.5×10^{-6} exclusion limit), using 500 mg of connective tissue leucine aminopeptidase (in 5 ml of Tris buffer, pH 8.0) purified up to the acetone step. The first fraction of activity, eluted at the exclusion volume of the column, suggested an apparent molecular weight of 1.5×10^6, a value much larger than ever reported for leucine aminopeptidase. Upon rerunning fraction I (Figure 9b) a redistribution of activity was observed. While it is not clear whether the large apparent molecular weight of this fraction is due to adsorption on some large protein molecules, there is no doubt that this fraction contains all the smaller molecular weight components. After rerunning fractions II and III it became equally clear that the small fractions can combine to form larger leucine aminopeptidase aggregates.

From this evidence, combined with that of the previous disc electrophoresis experiments, it seems reasonable to conclude that an equilibrium exists between smaller and larger aggregates of the enzyme and that the smallest functional unit consists of at least two units of differing net charge. Just how small the lowest unit might be is not clear but the following experiment suggests that 50,000 or even 25,000 molecular weight is possible. Using the same Agarose column, 3 ml of a mixture of blue dextran ($\sim 10^6$ molecular weight, bovine serum albumin, 70,000 molecular weight) and 1 mg of dansylated connective tissue leucine aminopeptidase was separated. The position of the dextran and bovine serum albumin is indicated by a solid line in Figure 9e. The dotted area depicts the fluorescence due to the dansylated leucine aminopeptidase. The main peak is at a position corresponding to a molecular weight of 100,000 while the trailing portion of the peak extends down to a position corresponding to 50,000 mol wt and lower. The enzyme–protein concentration was so low that no 280-mμ trace was produced. Under these conditions the smaller enzyme aggregates appear to predominate. In case the smallest subunit is 25,000 molecular weight, the lowest functional unit would be a tetramer and the predominant species of 400,000 molecular weight would contain 16 units.

Dissociation of Connective Tissue Leucine Aminopeptidase in Urea and p-Mercuribenzoate. The following experiment was performed in order to investigate how many subunits are present in the native leucine aminopeptidase molecule and if these units would migrate differently in an electric field. A 0.3% solution of purified connective tissue leucine aminopeptidase in 8.0 M urea and 0.1 M Tris buffer (pH 8.0) was kept at 40° for 16 hr without effecting any significant change in the disc electrophoresis pattern. Subsequently we determined that the enzyme was active following this treatment. Since that time we have detected through thin-layer chromatography and a new assay method based upon spectrophotometry (to be published) that the enzyme is active while dissolved in 8.0 M urea. The addition of 10^{-3} M p-mercuribenzoate and 10^{-3} M LiCl resulted in total loss of activity and a concomitant change of the disc electrophoresis pattern. Figure 10 shows the native and the denatured enzyme on gels which did not contain urea. Two bands are clearly distinguished. The slower band is not merely a precipitate since it has moved into the gel for about 1 mm. Combinations of the slow and fast bands could theoretically account for all the intermediate forms observed. In Figure 11a the native connective tissue enzyme was compared with a commercial hog kidney leucine aminopeptidase. The active bands of hog kidney enzyme are located between the small black bars to the right of Figure 11a. In Figure 11b,c the hog kidney leucine aminopeptidase and connective tissue leucine aminopeptidase are compared after denaturation. The gels used in this experiment were made from acrylamide
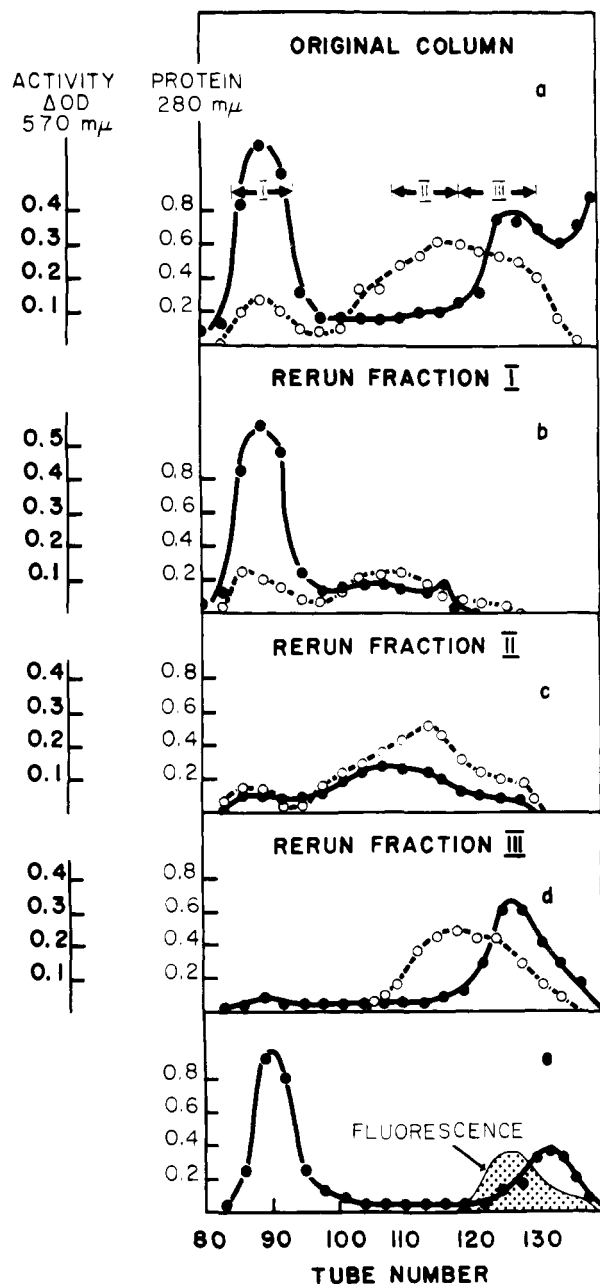
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FIGURE 9: Elution record of the acetone-precipitated enzyme. The experiments were performed on a 3 × 150 cm Agarose column as described in the section Agarose Chromatography. The connective tissue leucine aminopeptidase used in this experiment was purified up to the acetone fractionation step. Therefore protein peaks can be seen which are eliminated in the purest fraction (Figure 3) The sample (500 mg) was dissolved in 5 ml of 0.025 M Tris buffer (also the eluting buffer) containing 10% sucrose and applied to the column. The flow rate was kept at 0.4 ml/min by means of a Büchler micropump. Fractions (4 ml) were collected and assayed for the Leu-Leu hydrolyzing activity as described in the Materials and Methods section of the preceding paper. Roman numerals designate the fractions collected and subsequently concentrated to 5 ml each on an Amicon UM 1 filter (see text). For symbols, see legend of Figure 3.

solutions which were 8.0 M in urea. The first bands are traveling very fast and could be confused with tracer dye except that they do not diffuse. The speed of these

bands would be commensurate with a very low molecular weight. If the smallest unit of leucine aminopeptidase is 50,000 molecular weight and two polypeptide chains of differing size are present, the smallest chain might be well below 25,000 molecular weight. The slow band of connective tissue leucine aminopeptidase is still present indicating that it is not a precipitate formed on the urea-free gel (Figure 10). The kidney enzyme has, if any, only a very weak band in this region.

Isolation of Isozymes. The isolation of five active bands from acrylamide gel was greatly facilitated by the discovery that the dansylated derivative of connective tissue leucine aminopeptidase is fully active and that dansylation did not change the rate of migration of the enzyme in acrylamide gel at pH 8.1. This was verified by running fluorescent and untreated enzyme together on a single gel. Unequivocal evidence regarding the identity of the enzyme band could thus be obtained by excising fluorescent leucine aminopeptidase bands followed by a test for activity. By this method the result depicted in Figure 4 has been verified so that unequivocal proof of the identity of activity and protein was obtained.

Using a slab-type electrophoresis apparatus (E-C Apparatus Corp.) and a discontinuous buffer together with the fluorescent marking system it became possible to obtain sufficient isozyme material for a preliminary study of amino acid contents and the peptide pattern obtained after trypsin digestion. The material was recovered by the following procedure. A wide slot was formed in the top portion of the vertical gel slab from which a 5-mm wide chamber was subdivided on either side using a narrow strip of celloloid. The small chambers were filled with dansylated leucine aminopeptidase while the main chamber received untreated enzyme in sucrose. Electrophoresis was carried out at 100 mA and 400 V until the bromophenol blue had migrated 10–15 cm. The gel slab was removed from the running chamber and the enzyme containing gel excised between the fluorescent marker. The gel was mashed and forced through a syringe for further degradation. About 20 ml of pH 8.0 Tris buffer (10^{-3} M $MnCl_2$) was added and the mashed gel was dialyzed for 24 hr against Tris buffer (0.05 M). The gel fractions were then filtered and the dialysis was repeated. The filtrate contained isozymes which were concentrated on an Amicon UM 1 filter. All five bands showed activity by our regular assay method as well as by thin-layer chromatography.

Amino Acid Analysis. The enzyme bands isolated by semipreparative gel electrophoresis were precipitated with 10% trichloroacetic acid, dialyzed for 48 hr against two changes of 20 l. of distilled water, and lyophilized. About 2 mg of each band was hydrolyzed under vacuum at 110° for 24 hr in redistilled 5.7 N HCl. A Technicon single column (5.5 hr) system was used for the analysis. From Table II it may be seen that differences appear mainly between bands 2 and 3 vs. 0, 1, and 4, supporting the earlier suggestion that different chains do exist. Most striking is the difference in cystine content. Aromatic acids glycine and histidine are variable.

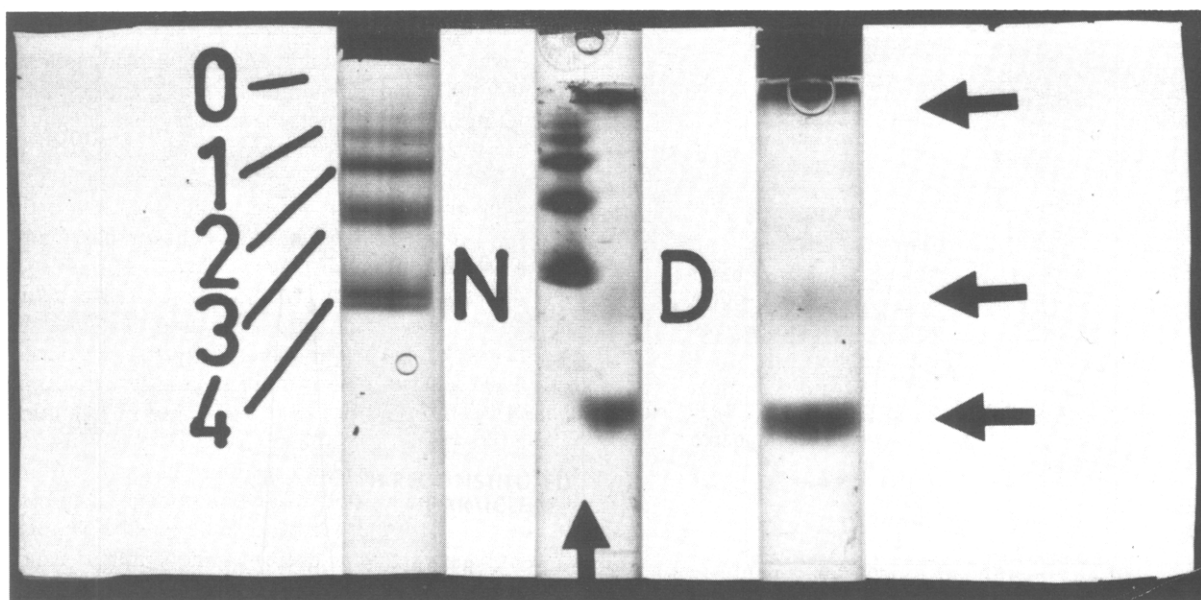The relationship of the isozymes to each other could

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FIGURE 10: The native and denatured forms of connective tissue leucine aminopeptidase are compared on gels not containing urea. Tubes from left to right: tube 1 = control, native enzyme tube 2 contains native (left) and denatured (right) leucine aminopeptidase. The right tube shows denatured enzyme only. The technique of running two samples on one gel is described by Clarke (1964) (see text for conditions).

FIGURE 11: Native connective tissue (left) and hog kidney enzyme are compared in tube a. Of the kidney enzyme only the bands between the small black lines are active. Tube b shows denatured kidney enzyme alone, tube c the denatured connective tissue enzyme alone. Tubes b and c are 8 M urea gels.
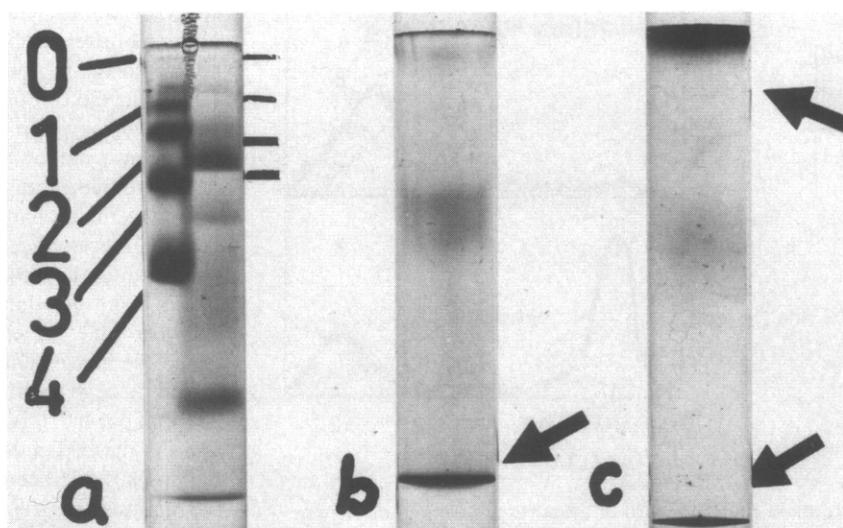
be too complex to be detected by this method. The possibility exists that, if A and B units are present and only A contains cysteine, either 1, 2, 3, or 4 A units combine with one B unit while the second isozyme could contain 3 B and 2 A units, for example. Simply adding up bands in Table II would not necessarily provide a lead to determine the amino acid composition of the subunits. The denatured bands will have to be examined to provide this information.

Peptide Maps of Connective Tissue Leucine Amino-peptidase. Tryptic digestion should produce similar peptide patterns from similar polypeptides. To further test the contention that leucine aminopeptidase contains two different chains the remainder of the dialyzed

trichloroacetic acid precipitated protein ($\sim$2 mg) was suspended in distilled water, crystalline trypsin (0.05 mg) in pH 8.0 Tris buffer was added, and the mixture was incubated for 3 hr at 40°. Thereafter an additional 0.02 mg of trypsin was added and the digestion was continued for an additional 2 hr. The digest was then concentrated to $\sim$50 μl on a Büchler Evapo-Mix and applied to a 16 $\times$ 22 in. Whatman No. 1 paper. Chromatography was carried out overnight with butanol–acetic acid–water (4:1:5, v/v). The papers were dried and subjected to high-voltage electrophoresis (Gilson Medical Electronics) at pH 3.7 in pyridine acetate buffer. Figure 12 shows the essential portions of the peptide maps photographed together for com-

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TABLE II: Amino Acid Analysis of Connective Tissue Leucine Aminopeptidase Isozymes.[a]

| | Amino Acid Residues (g/100 g of protein) | | | | |
| | Band Number | | | | |
Amino Acid	0	1	2	3	4
Aspartic acid	10.8	9.1	10.0	10.4	6.9
Threonine	7.2	5.6	6.4	5.9	5.4
Serine	12.1	12.8	*4.9*	9.3	11.4
Glutamic acid	19.5	19.3	16.2	15.0	18.4
Proline	3.5	3.9	4.7	5.2	4.8
Glycine	8.6	9.0	*3.9*	6.0	8.1
Alanine	6.1	6.3	4.8	6.0	5.7
Valine	4.2	5.0	5.0	5.0	4.8
Cystine	<1.0	<1.0	*5.1*	2.9	<1.0
Methionine	<1.0	<1.0	~1.0	<1.0	<1.0
Isoleucine	1.7	2.3	2.2	2.4	3.3
Leucine	4.9	4.5	6.5	4.8	4.6
Tyrosine	2.4	3.3	*7.9*	5.2	—
Phenylalanine	4.3	2.9	*7.1*	4.7	3.0
Lysine	6.5	7.7	9.9	8.1	9.8
Histidine	5.0	5.5	*2.6*	5.8	6.0
Arginine	3.4	3.1	3.0	5.0	6.0

[a] Italic values are those of largest deviation among the isozymes. The isozymes were isolated by semi-preparative disc electrophoresis on acrylamide slabs (see text).

parison. Dissimilarities are indicated by an arrow. The relative position of the spots could be checked by various color reagents. Ehrlich's reagent, for example, gave a good yellow "citruline" spot between two of the peptides. This clearly indicated that one of the two spots is missing in chromatogram 3 and 4. Significant is that the five leucine aminopeptidase isozymes are similar though not identical with respect to the trypsin peptides produced. This finding is consistent with the amino acid analysis and the disc electrophoresis data.

Based upon amino acid analysis, complete hydrolysis by trypsin should yield 15–20 peptides per 25,000 molecular weight subunit. The peptide map shows that between 10 and 15 peptides were obtained from the various isozymes.

Discussion

The purification of the leucine aminopeptidase activity from mammalian connective tissue has presented unusual problems. Apart from the well-known difficulties encountered in connective tissue work (mechanical resistance, scantness of cells, and low enzyme content) the enzyme appeared very labile in the crude extract. Dialysis with or without specific ions, lyophilization, and particularly ammonium sulfate caused irreversible inactivation. With the introduction of benzalkonium chloride as a precipitating agent the difficulties disappeared. The enzyme could now be precipitated with acetone and ammonium sulfate at room temperature. Since enzymes usually become labile with increasing purity we were tempted to attribute the increased stability to the long-chain aliphatic residues of benzalkonium chloride. The ultraviolet spectrum of the enzyme revealed traces of the benzene absorption
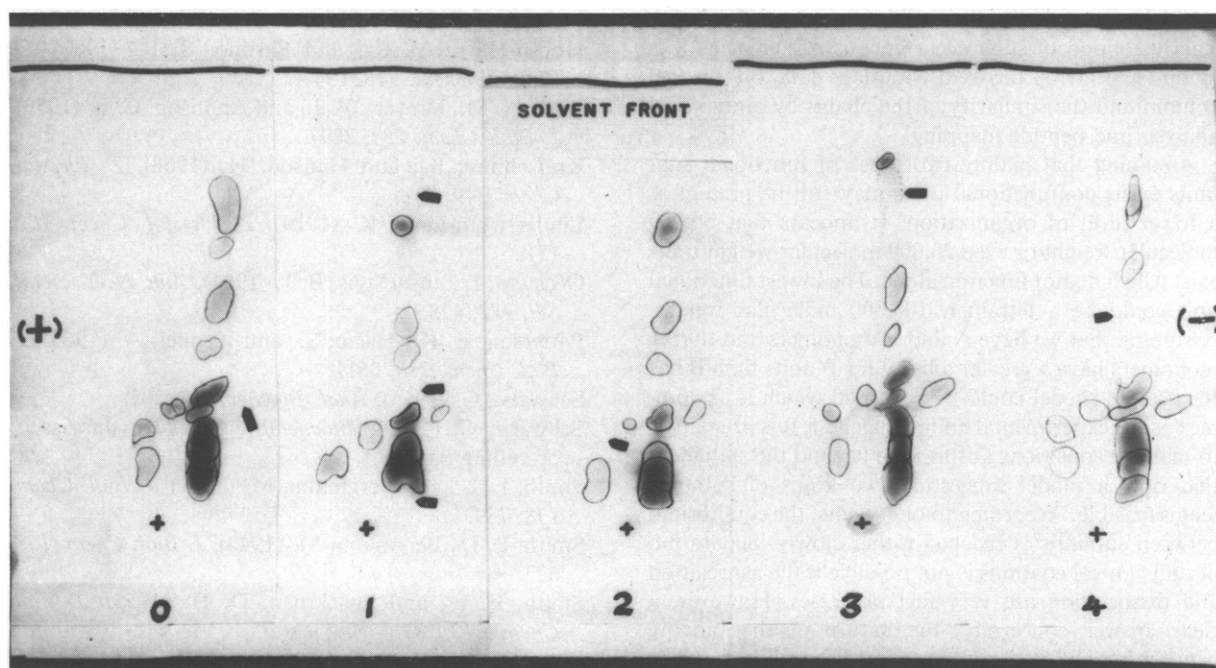

FIGURE 12: Peptide maps of the five isozymes of connective tissue leucine aminopeptidase. Arrows indicate points where peptides are missing. The numbers at the bottom of each chromatogram correspond to the band numbers on the gel (see Figure 10).

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typical for benzalkonium. It is doubtful, however, that the insensitivity of the enzyme to 8 M urea (activity persists in 8 M urea) can be explained by stabilization with benzalkonium.

The isozyme pattern of the connective tissue leucine aminopeptidase resembles that of lactate dehydrogenase. The latter also consists of two different forms apparently generated by two kinds of subunits. Our evidence certainly points to such system which has been suspected but never demonstrated to exist for a proteolytic enzyme. The amino acid analysis, the gel electrophoresis with native and denatured enzyme protein, the redistribution of activity after reelectrophoresis and rechromatography on Agarose leave little doubt about the involvement of different units. Whether both units are active or whether regulatory units are present remains an open question. The presence of such unit is in accord with the fact that the specific activity of the connective tissue enzyme is lower than that of the kidney enzyme. It also appears that the specific activities of the various aggregates of the connective tissue leucine aminopeptidase differ. We have not found any other factor yet which will increase one form of the enzyme or another although band 4 appears to become more prominent upon aging of the enzyme preparations. The system appears very complex and a careful study of this phenomenon is pending.

The structure of the hog kidney enzyme is less complex; nevertheless, four active areas could be detected on an acrylamide gel. Similar to the pulp enzyme, but more obvious, the fastest band contains most of the protein. The difference is more striking when denatured enzymes are compared by disc electrophoresis. It seems that kidney enzyme is composed of one kind of submit.

The generation of the various isozyme-like bands is possible through great affinity for an impurity which could be saturated with varying amounts of enzyme. The likelihood of such occurrence is not great as it is inconsistent with the redistribution data on the gel column and the similarity of the chains by amino acid analysis and peptide mapping.

Assuming that mainly two types of functional subunits exist, nonfunctional units may still be present at a lower level of organization. It appears that 50,000 molecular weight or even 25,000 molecular weight units exist which do not function alone. The lowest functional unit could be a tetramer, 100,000 molecular weight. Assuming that we have A and B monomers and that A monomers have a greater affinity for B units than B has for itself, a model could be proposed which is as complex as the experimental findings suggest. It is premature to make propositions at this time beyond the statement that only a model suggesting two kinds of subunits seems feasible. According to our results, the equilibrium between subunits is reached rather slowly. Separation of units on gel columns is not possible if the association and dissociation are very fast processes. However, a clear answer concerning the nature of the binding between the different units has not been obtained. The sensitivity of the leucine aminopeptidase to N-ethylmaleimide and p-mercuribenzoate suggests that cysteine might play a role in this process.

The connective tissue leucine aminopeptidase is a proteolytic enzyme as shown by its action on denatured α-chymotrypsin.[1] It is conceivable that such subunit structure may provide a mechanism for an allosteric type control of the enzyme activity, a very important aspect of cellular homeostasis. Less likely, but not impossible, is a generation of electrophoretically different bands due to errors in protein synthesis (substitutions) or due to genetic differences in species of animals from which the tissue was obtained. This, however, is not a satisfactory explanation for different size aggregates to occur as determined by exclusion chromatography.

The work of Kretschmer and Hanson (1968) which appeared while revising this manuscript was being revised is in excellent agreement with most of the results reported here. This indicates that the bovine lens leucine aminopeptidase has a structure very similar to the connective tissue leucine aminopeptidase. It is of interest that these authors suggest a molecular weight of 300,000–400,000 and the existence of 10 subunits. They also observed a large leucine aminopeptidase complex (10^6 g/mole).

Acknowledgments

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[1] No activity toward denatured mercuripapain was observed.