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Isolation and Purification of a Tissue Plasminogen Activator and Its Comparison with Urokinase*

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ABSTRACT: A simple procedure is described for the preparation from pregnant hog ovaries of a highly purified tissue plasminogen activator containing 100,000–175,000 tissue activator units/mg of protein. Tissue activator differs from the activator in urine (urokinase). Only within certain limits and under specified conditions is it possible to compare their activities in the same unitage. In fibrin plate assays one tissue activator unit was comparable to 0.1 CTA human urokinase unit. Gel filtration in glycine buffer at pH 2.35 indicated a molecular size of about 60,000 which is larger than the main component observed in urokinase preparations (mol wt 54,000). Though uniform by gel filtration, the tissue activator preparation could be further fractionated by zone electrophoresis. The active component had a mobility close to that of one of the active urokinase components and to that of bovine serum albumin. The properties of the tissue activator make it unlikely that it could be the source of plasminogen activator in urine.

The fibrinolytic activity of animal issues, originally thought to be caused by tissue proteases acting directly on fibrin (Macfarlane and Biggs, 1948), was later (Astrup and Permin, 1947, 1948; Astrup, 1951) found to be an indirect reaction involving the activation of a precursor, plasminogen, present in blood, to the active enzyme, plasmin, a trypsin-like protease (enzyme classification number 3.4.4.14). The tissue plasminogen activator is present in the microsomal fractions of tissues (Tagnon and Petermann, 1949; Lewis and Ferguson, 1950) and in lysosomes (Lack and Ali, 1964). Data on the assay of tissue plasminogen activator, its distribution in tissues, and its possible physiological significance have been reviewed recently (Astrup, 1966). Activators of plasminogen are also present in blood and urine. The human urinary activator (urokinase) has been prepared to a high degree of purity (Lesuk *et al.*, 1965; White *et al.*, 1966).

Purification of tissue plasminogen activator has been hampered because of its strong adherence to particulate cellular material from which it could not be extracted with solutions usually applied for such purposes. The observation that tissue plasminogen activator can be brought into solution by molar thiocyanate (Astrup and Stage, 1952) and separated by acid precipitation (Astrup and Sterndorff, 1956) led to a method for the quantitative assay of tissue plasminogen activator (Astrup and Albrechtsen, 1957). A preparation made from pig heart tissue served as a reference standard. Later, Bachmann and Sherry (1963) observed that

* From the James F. Mitchell Foundation, Institute for Medical Research, Washington, D. C. 20015. *Received August 23, 1968*. Supported by Grant HE-05020 from the U. S. Public Health Service, National Institutes of Health, National Heart Institute.

† Recipient of postgraduate fellowship from the Technical University, Copenhagen, Denmark, during the early part of this study.

tissue plasminogen activator could be extracted selectively with an acetate buffer at pH 4.2 and further purification led to preparations (Bachmann *et al.*, 1964) approaching in fibrinolytic activity those of highly purified urokinase preparations.

Preparation of highly potent tissue plasminogen activator in amounts required to elucidate its biochemical and physiological properties depends upon the availability of a source more rich in tissue plasminogen activator than porcine heart and of simple purification methods. It is the purpose of this communication to contribute to the solution of these problems. Hog ovaries were used as a potent source of tissue plasminogen activator. Simple procedures were devised, yielding highly potent preparations of tissue plasminogen activator which were found to be chemically different from urokinase. Preliminary data from this study have been briefly reported (Astrup and Kok, 1965; Kok and Astrup, 1967).

Materials and Methods

Fresh pig hearts were collected from slaughter houses. Frozen hog ovaries were first obtained through the Warner-Lambert Research Institute, Morris Plains, N. J., and later through direct arrangements with slaughter houses and packing plants. Chemicals were reagent grade unless otherwise stated. Bovine thrombin (48 N.I.H. units/mg) was from Leo Pharmaceuticals, Copenhagen, Denmark. Bovine fibrinogen was prepared by $(NH_4)_2SO_4$ precipitation (Astrup and Müllertz, 1952) with the improvements introduced by Brakman (1967). The following samples of human urokinase were available for comparison: Sterling-Winthrop preparation Tr-S-143 marked 37,600 CTA urokinase units/unit of optical density at 280 mμ (calculated to correspond to approximately 50,000 CTA units/mg of protein assuming equal specific optical density of urokinase and contaminating impurities) and the molecular weight reported as 54,000; Sterling-Winthrop preparation Tr-T-101 marked 73,500 CTA units/mg of protein; Abbott preparation 2517-110C marked 50,000 CTA units/mg of protein; Leo preparation 66021 marked 10,000 Ploug urokinase units/vial (with approximately 1.3 mg/vial yielding about 10,000 CTA units/mg); Leo preparation 161071 marked 4200 Ploug units/mg (equal to about 5500 CTA units/mg). For standardization a CTA urokinase reference standard from the Committee on Thrombolytic Agents (advisory to the National Heart Institute) was available through Dr. Allan J. Johnson, New York.

Activator was assayed by the fibrin plate method (Astrup and Albrechtsen, 1957) with 0.1% bovine fibrinogen clotted with bovine thrombin. Excessive amounts of salts or other compounds introduced by purification procedures have relatively little influence on assays by the fibrin plate method because clot formation occurs before application of the samples and because these can be applied in highly diluted form. The accuracy of the determinations is ±7%. Solutions and serial dilutions of activator for fibrin plate assays were prepared in 1 M potassium thiocyanate containing 0.25% gelatin (bacteriological grade). Purified activator preparations were also assayed by the lysis time method of Lassen (1958), with an estimated accuracy of ±1%, though requiring solutions 100 times more concentrated than the fibrin plate assay. Activator concentrations were estimated from the double logarithmic, linear activity curves by interpolation on a dilution curve prepared with an appropriate reference standard simultaneously assayed. Preparations of tissue plasminogen activator for standardization were made from pig heart as described by Astrup and Albrechtsen (1957), and assayed against a reference standard kept in the laboratory. Tissue activator concentrations were expressed in Astrup and Albrechtsen units (A and A units), defined originally as the amount of tissue activator present in 1 mg of an arbitrarily selected preparation from pig heart muscle. Concentrations of urokinase were expressed in Ploug units (Ploug and Kjeldgaard, 1957) or in CTA units (as defined by the Committee on Thrombolytic Agents, advisory to the National Heart Institute, U. S. Public Health Service). In several experiments, using different preparations of urokinase, we found the mean conversion factor estimated by the fibrin plate method and the lysis time method to be 0.76 and 0.77 Ploug unit, respectively, per CTA unit. It is not possible to obtain an accurate conversion factor permitting the expression of tissue plasminogen activator assays in urokinase units. Beyond certain concentration limits, curves obtained with tissue plasminogen activator and urokinase deviate from each other. In urokinase assays by the fibrin plate method the activity curves deflect downward when the lysed areas drop below about 200 mm^2 in diameter product. Traces in the fibrinogen of inhibitor reacting chiefly with urokinase could be responsible for this deviation. Activity curves obtained with tissue plasminogen activator prepared from different organs or species do not show such deviations. These differences have been observed regularly and preclude the exact quantitation of tissue plasminogen activator and urokinase in terms of the same unitage except within a limited range of activity and under specified conditions of assay. In the fibrin plate method one A and A tissue activator unit equalled in activity 0.076 Ploug unit or 0.10 CTA unit of urokinase. Unless otherwise stated, all activities reported below are in A and A units.

Progress of purification was ascertained by nitrogen determinations (micro-Kjeldahl after Ma and Zuazaga, 1942) of preparations after removal of low molecular weight components in appropriate samples by dialysis overnight against running tap water.

Particle size was determined by gel filtration at pH 2.35 in 0.1 M glycine-HCl buffer containing 0.2 M KCl. In each experiment calibration was performed with four or more of the following proteins assuming the molecular weights mentioned: cytochrome *c*, from horse heart (Sigma Chemical Co., 13,000); soy bean trypsin inhibitor, crystalline (Novo Laboratories, 24,000); pepsin, 1:60,000, crystalline (Sigma, 35,000); ovalbumin, five-times crystallized (Pierce Chemical Co., 45,000); bovine albumin, Cohn fraction V (Sigma,

67,000); and bovine γ-globulin, Cohn fraction III (Sigma, 160,000). Solutions, containing 5 mg of reference protein per ml or from 0.25 to 5 mg of activator protein per ml, were centrifuged at 10,000*g* for 15 min and the supernatants (2 ml) were applied to the column. Each solution was separately applied with sufficient time intervals to avoid overlapping of solute peaks. The ultraviolet absorbancy of the effluent was monitored continuously using a Beckman DB spectrophotometer with microaperture flow cell and programmed scanning accessory. Recording was automatic, either continuously or when appropriate, at 10-min intervals. The fraction number was counted, and the time and effluent volumes were measured, from the moment half the sample volume had entered the gel. End points were read at maximum solute concentrations passing through the flow cell as determined by extrapolating to an apex of a solute peak on the elution diagram. As a further check, reference proteins with molecular weight close to those expected for the activators were run between activator solutions. Calibration diagrams were plotted with time, effluent volume, or number of fractions against logarithms of molecular weights.

Gel electrophoresis was conducted vertically on an apparatus resembling that of Smithies (1959). Starch gels were prepared with 13 g of hydrolyzed starch (Connaught Laboratories) per 100 ml of 0.05 M glycine-HCl buffer at pH 2.35. Acrylamide gels were prepared from solutions of 15 or 20 g of Cyanogum 41 (American Cyanamide Co.) and 0.4 ml of tetramethylethylenediamine in 300 ml of water, polymerization being induced with 0.4 g of ammonium persulfate. After 2 hr the gel was equilibrated for 6 hr with the glycine buffer. Applied samples contained approximately 1 mg of protein in 50 μl of glycine buffer. The buffer was circulated between the compartments. After completion of electrophoresis gels were removed and sliced through their midplane. One-half was stained with Naphthol Blue Black (0.55% in aqueous methanol with acetic acid). The other half was cut into 1-cm wide strips through the sample wells, and then divided into 2-mm sections. Each section was suspended in 2 ml of 0.05 M sodium barbital buffer (pH 7.75) containing 0.25% gelatin and 1 M KSCN, and the supernatant assayed on fibrin plates, after appropriate dilution with the same buffer.

Results

Preparation of Tissue Activator. In the development of simple methods for the preparation and purification of tissue plasminogen activator, numerous procedures have been tried, details of which will not be included here. An important step was the selection of pregnant hog ovaries as starting material because of their high activities. Normal hog ovaries were found to contain from 500 to 5000 A and A units per g of fresh tissue. Ovaries from pregnant hogs regularly contain 30,000, and occasionally 50,000, units per g, the highest concentrations so far recorded for any organ or species. Briefly described, the finally adopted procedure consists of the following steps.

1. EXTRACTION OF ACTIVATOR. Frozen ovaries (1 kg), partially thawed, were passed through a meat grinder in the cold room, briefly homogenized with 0.05 M KCl in a Waring blender, and then diluted with a total of 10 l. of cold 0.05 M KCl, adjusting the pH to 6.0 with 1 M HCl. Stirring for 2 hr in the cold followed by centrifugation gave a supernatant with only 3–5% of the total activity. The active sediment was stirred overnight at room temperature with 10 l. of 2 M ammonium thiocyanate, adjusting the pH to 7.3 with 1 M KOH. After centrifugation, the solution (*ca.* 10 l.) contained per ml about 3000 units and 0.6 mg of protein nitrogen, equal to about 5000 units/mg of nitrogen. Total yield was $\sim 30 \times 10^6$ units per kg of ovaries.

2. ACID TREATMENT. After dilution with one volume of water and addition of 1 M HCl to pH 2.5, an active precipitate was separated by centrifugation and stored overnight at −20°. The precipitate was then thawed and treated for 1 hr with 1.5 l. of 1 M ammonium thiocyanate at pH 5.9. The supernatant, separated by centrifugation, was again adjusted to pH 2.5, and the resulting precipitate treated twice with 800 ml of cold ethyl ether, with intermittent freezing below −20°, to remove lipid material. After treatment with 2000 ml of 1 M ammonium thiocyanate at pH 5.9 the supernatant (*ca.* 2,000 ml) contained approximately 10,000 units and 0.5–0.6 mg of nitrogen/ml, equal to 17,000–20,000 units/mg of nitrogen. The yield per kg was $\sim 20 \times 10^6$ units. A considerable loss in total activity occurred during this step but a large amount of inert material was removed.

3. ZINC FRACTIONATION. The extract (2000 ml) was added to 2000 ml of a solution containing 0.2 M $ZnCl_2$ and 0.6 M glycine, keeping the pH at 8 to prevent precipitation. Adjusting the pH to 5.8 produced an active sediment which, after 0.5 hr at room temperature, was separated by centrifugation, suspended in a solution containing 0.02 M $ZnCl_2$ and 0.06 M glycine at pH 5.8, and again centrifuged. The precipitate was then dissolved in 1000 ml of a solution of 0.01 M $ZnCl_2$ and 0.03 M glycine at pH 4.3, leaving an inactive sediment. The final solution, *ca.* 1000 ml, contained approximately 18,000 units and 0.3–0.5 mg of nitrogen/ml, equal to 35,000–60,000 units/mg of nitrogen. The yield per kg was $\sim 18 \times 10^6$ units.

4. PRODUCT I. Adjusting the pH to 5.8 produced again an active precipitate which, separated by centrifugation, was treated first with 0.05 M glycine at pH 5.8, then by 0.05 M KCl, and finally with acetone and ether, yielding about 1.5 g of a grayish powder with about 10,000 or 70,000 units per mg of nitrogen. The yield per kg was $\sim 15 \times 10^6$ units. Product I is stable for prolonged periods. It is soluble at acid reaction at low salt concentrations. In saline–barbital buffer at pH 7.75 concentrations of 30,000 units/ml can be obtained.

5. GEL FILTRATION. The brownish solution (15 ml) containing 25 mg (*ca.* 250,000 units) of product I/ml of 0.1 M glycine-HCl buffer at pH 2.35 was applied to a 90 × 2.5 cm Sephadex G-200 column. Separation was achieved with the same buffer containing, in addition, 0.2 M KCl by ascending flow at a rate of 2–2.5 ml/cm² per hr. Effluents were collected at 30-min

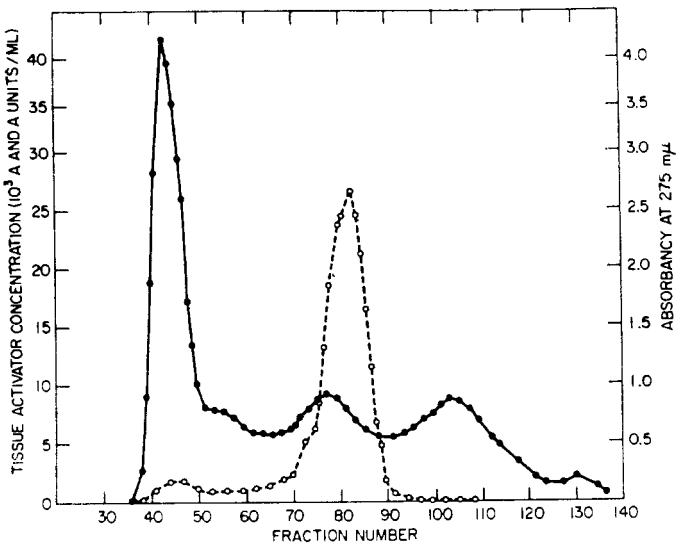

FIGURE 1: Fractionation of tissue activator product I on Sephadex G-200 in 0.1 M glycine-HCl buffer (pH 2.35) containing 0.2 M KCl. Column size, 90 × 2.5 cm; sample size, 15 ml; ascending flow rate, 2 ml/cm² per hr. (●—●) Ultraviolet absorbancy at 275 mμ. (O- - -O) Tissue activator concentration in A and A units per milliliter.

intervals. Ultraviolet absorbancies were measured at 275 mμ. Figure 1 shows a gel filtration pattern typical for product I. The first protein peak, representing the major part, was nearly inactive. The active fraction appeared as the slower part of another much smaller peak. This was followed by an additional peak of inactive material.

6. PRODUCT II. The strongly active fractions (76–87 in Figure 1) were pooled and dialyzed against 0.01 M glycine-HCl buffer at pH 2.35 containing 0.01 M KCl.

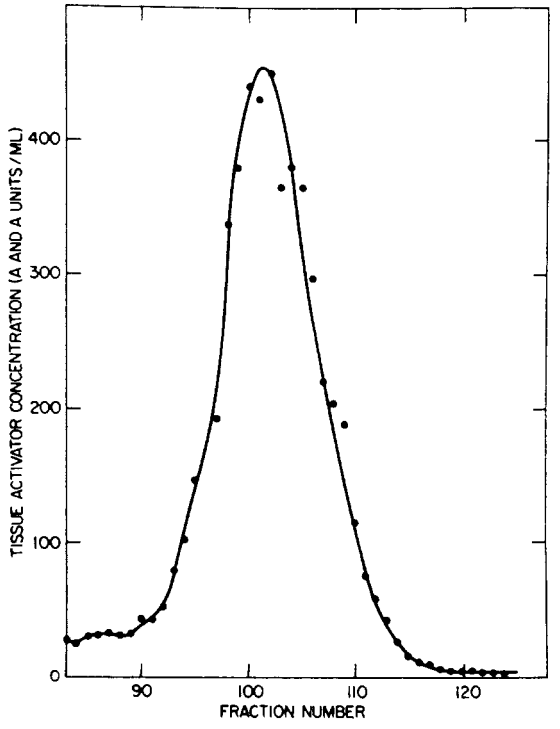

FIGURE 2: Gel filtration of tissue activator product II on Sephadex G-200 in 0.1 M glycine-HCl buffer (pH 2.35) containing 0.2 M KCl. Sample size, 0.5 mg in 2 ml; ascending flow rate, 1.5 ml/cm² per hr.

Adjusting the of pH to 7.1 in the cold with dilute KOH produced a precipitate which was separated by centrifugation and treated with acetone and ether. Batches prepared in this manner contained from 100 to 175 × 10^3 units per mg of protein in yields of 50–65% of product I, corresponding to 8–10 × 10^6 units per kg of ovaries.

Comparison of Tissue Activator and Urokinase. 1. MOLECULAR SIZE. Lesuk *et al.* (1965, 1966) prepared urokinase with a specific activity of 104,000 CTA units/mg of protein and mol wt 54,000. White *et al.* (1966) reported two different types of urokinase: one with 218,000 CTA units/mg of protein by a fibrinolytic assay (or 170,000 CTA units by an esterase assay on synthetic substrates) and mol wt 31,500; the other with 93,000 CTA units/mg (fibrinolytic assay) and mol wt 54,700.

At room temperature (about 22°) product II (0.3 mg/ml) gave an approximately symmetrical activity curve (Figure 2) resembling the activity peak in the purification diagram (Figure 1). The slight irregularities in the determinations in the activity curve in Figure 2 are considered insignificant since they appear in a random manner in different experiments and are mostly within the range of the error of estimation which increases when solutions low in protein concentration are handled. Calculated from the effluent volume or number of fractions, the particle size of tissue plasminogen activator was about 60,000. The active peak of product I (5 mg/ml) gave in similar experiments a particle size of 57,500. Gel filtration in the cold (4°) of product II (2.5 mg/ml) gave a slightly larger particle size (62,000–63,000). The ultraviolet absorbancy paralleled the activity suggesting that further purification of product II could not be based on separation according to differences in molecular sizes.

Gel filtration curves obtained with urokinase are shown in Figure 3. The activity peaks are less symmetrical than with tissue plasminogen activator. This could possibly be caused by adsorption of urokinase to the gel but additional active compounds of smaller

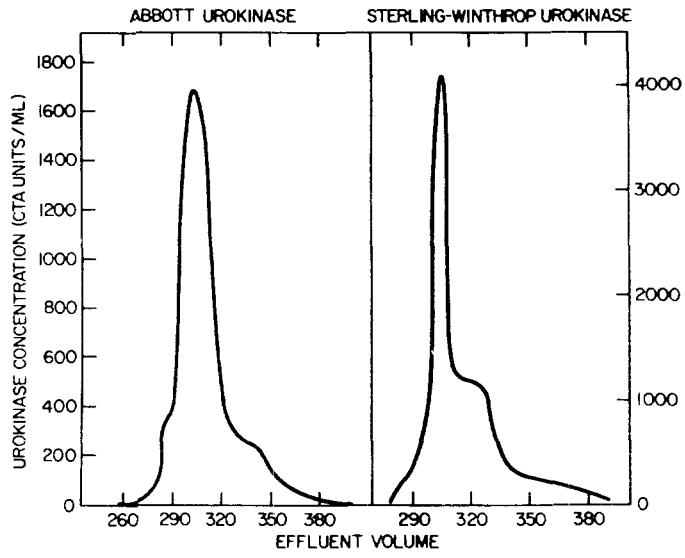

FIGURE 3: Gel filtration of Abbott (2517-110C) and Sterling-Winthrop (Tr-S-143) urokinase on Sephadex G-200. Conditions as in Figure 2. Samples: 255,000 CTA units of 2517-110C in 1.7 ml; 226,000 CTA units of Tr-S-143 in 2 ml.

molecular sizes could also be present. At 4° the asymmetry decreased. The main urokinase peaks gave particle sizes of about 54,000 for the Abbott as well as the Sterling-Winthrop preparations, comparing well with those reported for a different set of conditions (Lesuk *et al.*, 1965, 1966; White *et al.*, 1966). Results with less pure preparations suggested that more than one molecular species of urokinase could be involved. Thus, in Leo preparation 66021 two active peaks corresponded to molecular sizes of about 54,000 and 43,000 (Figure 4).

2. ZONE ELECTROPHORESIS. On starch gel tissue plasminogen activator (product II) separated into three distinct bands, only the fastest (marked by arrows) being active (Figures 5A-II and 6A-II). Similarly tested, product I produced an active band migrating with the same rate within a broad, diffuse zone (Figure 5A-I). Other tissue plasminogen activator preparations (product II) prepared from four pooled intermediate products separated into two active fractions (Figures 5B-III and 6B-III).

The active peaks produced on starch gel by highly purified urokinase did not coincide with maximum stainability. The most pure Leo sample (66021) (Figure 5B-I) and the Sterling-Winthrop sample (Tr-T-101) (Figure 5B-II) showed only one active peak migrating at a rate different from the tissue plasminogen activator peaks (Figure 5B-III). The Abbott sample (2517-110C) showed three active peaks (Figures 5B-IV and 6B-IV), of which the middle peak appeared at the same location as one of the tissue plasminogen activator peaks. The slowest moving active component did not stain. The less pure Leo sample (161071) (Figure 5A-III) showed two active peaks, the slowest being at the same location as the active component in the tissue plasminogen activator preparation (Figure 5A-II).

On acrylamide the pure urokinase preparations showed one active peak located in the main band (Figures 5C-I, III, IV). The active components in the urokinase preparations migrated slightly faster than the active fraction of tissue plasminogen activator product II (Figure 5C-II) and at approximately th same rate as bovine serum albumin (Figure 5C-V) There was less separation on acrylamide than on starch gel.

Discussion

The results show that it is possible by selecting an appropriate source of material (pregnant hog ovaries) to devise relatively simple procedures for the preparation of highly potent preparations of tissue plasminogen

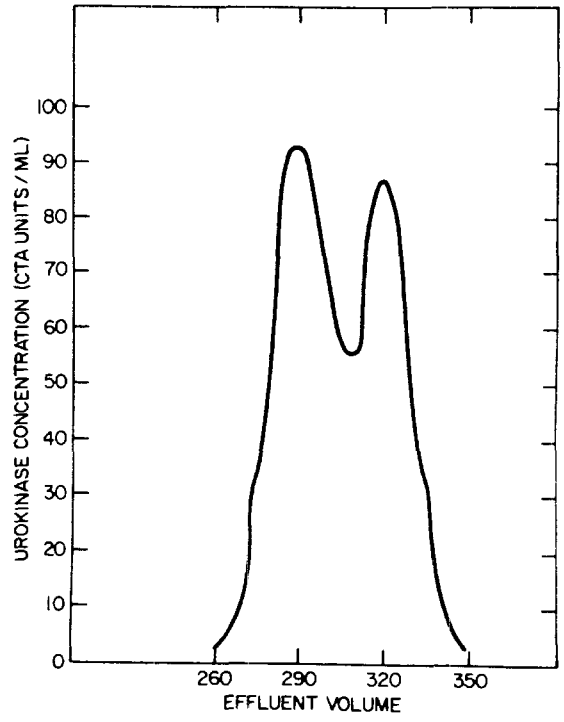

FIGURE 4: Gel filtration of Leo urokinase (66021) on Sephadex G-200. Conditions as in Figure 2. Sample, 9000 CTA units in 2 ml.

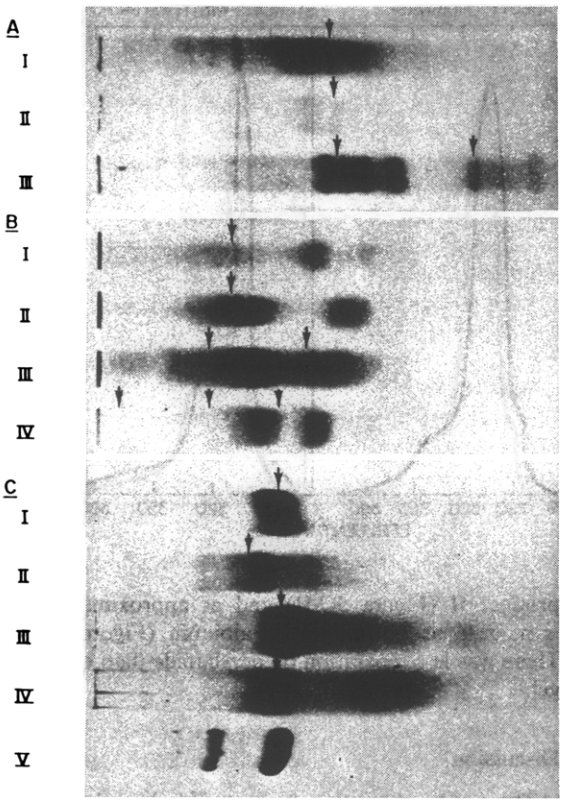

FIGURE 5: Electrophoresis patterns on starch gel and acrylamide gel in 0.05 M glycine-HCl buffer (pH 2.35). Peaks of activity are marked by arrows. (A) Starch gel, 1.7 V/cm, 16 hr, 22°. (I) Tissue plasminogen acivator product I; (II) tissue plasminogen activator, product II; and (III) Leo urokinase (161071). (B) Starch gel, 1.3 V/cm, 18 hr, 4°. (I) Leo urokinase (66021); (II) Sterling-Winthrop urokinase (Tr-T-101); (III) tissue plasminogen activator product II, prepared from pooled product I; and (IV) Abbott urokinase (2517-110C). (C) 6.5% acrylamide gel, 2 V/cm, 16 hr, 4°. (I) Abbott urokinase (2517-110C); (II) tissue plasminogen activator, product II, prepared from pooled product I; (III) Sterling-Winthrop urokinase (Tr-T-101); (IV) Leo urokinase (66021); and (V) bovine albumin.

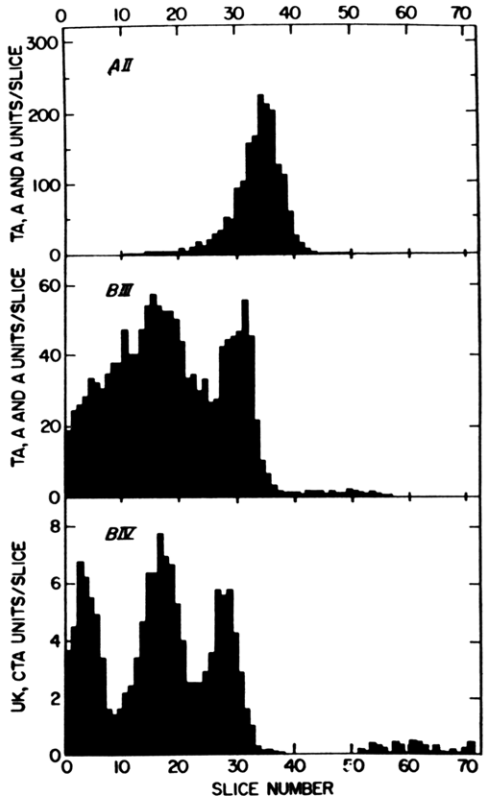

FIGURE 6: Activator concentrations in electrophoresis patterns in Figure 5. (A-II) Tissue plasminogen activator product II (Figure 5A-II); (B-III) tissue plasminogen activator, product II, pooled (Figure 5B-III); and (B-IV) Abbott urokinase (Figure 5B-IV).

activator. These preparations, as well as the available urokinase preparations, show a uniform gel filtration pattern with their activity peaks in a narrow range of particle size and with only little contamination by products of different sizes. However, the results of zone electrophoresis show: (1) that these highly purified preparations are still not electrophoretically uniform, (2) that the activity does not necessarily follow the main peaks of protein material, and (3) that in some of the preparations activator activity is located in more than one peak.

Estimated by gel filtration, the particle size of tissue plasminogen activator corresponded to a molecular weight of 60,000. Slight differences observed at different concentrations and temperatures (22 and 4°) could possibly be caused by adsorption to the gel, or by changes with temperature in the tertiary structure of the molecules. Interestingly, Bachmann *et al.* (1964) had obtained a distribution coefficient, K_d, of 0.28 by gel filtration of their purified tissue plasminogen activator from pig heart with K_d values for human albumin monomer (mol wt 68,000–69,000) and cyanmethemoglobin, respectively, at 0.26 and 0.32. The unexpected high value for cyanmethemoglobin could possibly be explained by dissociation into subunits under their experimental conditions (Andrews, 1964). Assuming a molecular weight between 40,000 and 46,000 for cyanmethemoglobin (Andrews, 1964; Winterhalter, 1966) interpolation on a log mol wt *vs.* K_d diagram would for $K_d = 0.28$ yield a particle size for tissue plasminogen activator of 56,000 to 60,000, which compares well with our determinations. The only other molecular weight determination is a value in the region of 50,000, estimated by gel filtration for a preparation from rabbit kidney (Ali and Evans, 1968). A plasminogen activator isolated from kidney cell cultures was reported to resemble urokinase more closely than tissue activator (Painter and Charles, 1962).

For urokinase Lesuk *et al.* (1965, 1966) found a molecular weight of 53,000–54,000 by ultracentrifugation and gel filtration of preparations with specific activities of about 100,000 CTA units/mg of protein. White *et al.* (1966) reported two active components in their preparations: one of mol wt 54,700 determined by ultracentrifugation, thereby comparing well with the data of Lesuk *et al.* (1965, 1966) as well as with ours, the other of mol wt 31,500. The latter reached a specific activator activity of 218,000 CTA units/mg and an activity comparable with 170,000 CTA units/mg

assayed on synthetic substrate. Recently, Lesuk *et al.* (1967) reported that tryptic digestion produced active fragments with a molecular weight about 36,000 and a specific esterase activity of about 175,000 CTA units/mg, suggesting that such components could result from proteolytic degradation of native urokinase. We found no indication of components with molecular sizes of 32,000–36,000 in the urokinase preparations studied. The slightly asymmetric peaks of the Abbott or Sterling-Winthrop preparations could suggest a minor contamination with active fractions of only slightly lower molecular size than the main peak. In the Leo preparation (66021) the two active peaks corresponded to molecular weights of about 54,000 and 43,500. It should be noticed that our determinations were made in a glycine-HCl buffer at pH 2.35, where purified activator preparations are quite stable, and where hydrogen bonding would be minimal. White *et al.* (1966) observed identical molecular weights at different pH values by ultracentrifugation. Burges *et al.* (1965) studied urokinase (Leo Pharmaceuticals, 7000 Ploug units/mg) by gel filtration at pH 7.5 in Tris buffer containing 0.1 M KCl. They found a molecular weight of 34,500 and reported an active contaminant with a molecular weight above 100,000. Doleschel and Auerswald (1967) studied a concentrate from normal urine by gel filtration at pH 8.2 in 0.1 M Tris buffer with 1 M NaCl. They observed a wide distribution of activator activity over fractions of different sizes except the smallest. Several subfractions could be separated with activity peaks corresponding to molecular weights around 27,000, 54,000, and 104,000. The urine was concentrated by evaporation under vacuum and chromatographed at room temperature at pH 8.2. It is quite possible that degradation could occur under such conditions. Hamberg and Savolainen (1966), using gel filtration at room temperature, compared fresh urine concentrated by cold ultrafiltration with urokinase (Leo Pharmaceuticals) and observed molecular weights close to human serum albumin.

Tissue activator, highly purified, is of uniform molecular size with a molecular weight about 60,000, which differs from those found for urokinase. Zone electrophoresis revealed the preparations to be heterogeneous and amenable to further purification, possibly three to five times. This would bring the specific activity within the range of the most pure urokinase preparations. The different migration rates confirmed the chemical differences between tissue plasminogen activator and urokinase, as indicated by their behavior in the assays, and by the different susceptibility to inhibitors (Brakman *et al.*, 1966).

The results would suggest that tissue plasminogen activator and urokinase would differ in their behavior in the organism; they could serve to explain differences in reactions with inhibitory agents, and they bear upon speculations about the origin of urokinase in the body. Thus the particle size of tissue plasminogen activator makes it doubtful that it could normally pass through the kidney and appear in the urine, suggesting a different origin for urokinase. Though the circulating blood may contain different plasminogen activators, derived either from tissues or by activation in the blood, the particle size of urokinase would also indicate that it could not normally originate in the blood. The urokinase fractions with reported molecular weights around 30,000 could possibly be degradation products formed during processing of the urine, and they would therefore not represent native urokinase. The possibility that the observed differences between the porcine tissue activator and the human urokinase could be due to species differences should be kept in mind, although we consider this remote, since deviations suggesting such differences were not observed in assays of activator preparations made from different organs or animal species. With the simple procedures now available for the preparation of highly purified tissue activator, it should be possible to attack many problem in the biochemistry and physiology of fibrinolysis.

Acknowledgments

We thank Dr. Robert L. Kroc and Mr. John Doczi of the Warner-Lambert Research Institute for their help in providing frozen ovaries from pregnant hogs. Urokinase was generously supplied by Dr. Alex Lesuk, Sterling-Winthrop Research Institute, Rensselaer, N. Y., Dr. Milton M. Mozen, Abbott Laboratories, North Chicago, Ill., and Dr. Ole Bremer, Leo Pharmaceuticals, Copenhagen, Denmark.

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Beef Liver Esterase as a Catalyst of Acyl Transfer to Amino Acid Esters*

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ABSTRACT: Purified beef liver esterase has been shown to be an efficient catalyst in the transfer of acyl groups from the methyl esters of suitable amino acids (*e.g.*, L-phenylalanine) and fatty acids (*e.g.*, β-phenylpropionic acid and hexanoic acid) to the α-amino group of L-phenylalanine methyl ester and of other amino acid esters at pH 7.2–8.5. Although D-phenylalanine methyl ester is readily hydrolyzed by the enzyme, it is relatively ineffective as an acceptor of acyl groups from the methyl esters of L-phenylalanine and β-phenylpropionic acid. With L-phenylalanine methyl ester as the acceptor amine, the acylamino acid ester initially formed (Phe-Phe-OMe, β-phenylpropionyl-Phe-OMe, or hexanoyl-Phe-OMe) is hydrolyzed to the acylamino acid (Phe-Phe, β-phenylpropionyl-Phe, or hexanoyl-Phe), in accordance with the side-chain specificity of the enzyme with respect to the acyl donor in hydrolytic or transfer reactions. On the other hand, with L-isoleucine methyl ester or glycine methyl ester as the acceptor amine, the acylamino acid ester that is formed is not converted to a detectable extent to the corresponding acylamino acid; this finding is consistent with the relative resistance of these two amino acid esters to the hydrolytic action of beef liver esterase. For the quantitative estimation of the reaction components that contain an acyl group derived from a fatty acid ester, analytical gas–liquid partition chromatography was employed. A detailed study has been made of the reaction in which L-phenylalanine methyl ester serves both as the acyl donor and the acceptor amine, with respect to the influence of initial substrate concentration, enzyme concentration, pH, and the presence of an organic solvent (dioxane). By quantitative estimation of the components of the reaction mixture, using automatic spectrophotometric monitoring of effluents from a Sephadex G-10 column, it has been possible to define the effect of these variables on the partition of the phenylalanyl units of L-phenylalanine methyl ester between hydrolysis to free amino acid and the synthesis of Phe-Phe-OMe and Phe-Phe. In connection with these studies, the kinetic parameters were determined for the action of beef liver esterase at pH 7.2–8.5 on an extensive series of fatty acid methyl esters. These data served as a basis for the selection of methyl β-phenylpropionate and of methyl hexanoate as potential acyl donors to amino acid esters. Comparison of the kinetic data with those reported by other investigators for purified liver esterase preparations provides additional evidence for the view that the ability to transfer suitable acyl units to the α-amino group of L-amino acid esters is an intrinsic property of beef liver esterase.

In a previous communication from this laboratory, it was reported that an enzyme preparation (from beef liver) effective in the hydrolysis of esters such as methyl butyrate and L-leucine methyl ester also is an efficient catalyst in the synthesis of peptides from suitable amino acid esters (Krenitsky and Fruton, 1966). Indeed, in its action on 50 mM L-phenylalanine methyl ester at pH 7.5, the enzyme preferentially catalyzes the formation

* From the Departments of Biochemistry and Biology, Yale University, New Haven, Connecticut 06520. *Received September 12, 1968.* These studies were aided by grants from the National Institutes of Health (GM-06452) and from the National Science Foundation (GB-5212X).

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