Studies on the Tissue Activator of Plasminogen

DISTRIBUTION OF ACTIVATOR AND PROTEOLYTIC ACTIVITY IN THE SUBCELLULAR FRACTIONS OF RABBIT KIDNEY

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1. On subcellular fractionation of rabbit kidney by differential and density-gradient centrifugation, a high proportion of the tissue activator of plasminogen activity was found to be particulate and displayed sedimentation properties associated with the lysosome-rich fraction as judged biochemically by the acid-phosphatase activity. 2. The activator activity is closely associated with a latent protease whose activity is enhanced in the presence of Triton X-100 or sodium deoxycholate in the neutral pH range. Besides hydrolysing casein this protease is also capable of attacking fibrinogen at pH 7.4. 3. The pH optimum for activator activity and its inhibition by ϵ -hexanoic acid (ϵ -aminocaproic acid) point to its possible similarity to urokinase, an activator of plasminogen present in the urine of most mammals.

Extracts of human and animal tissues made with water, 0.9% sodium chloride and thiocyanate solutions are known to contain an activator capable of converting the inactive blood proenzyme plasminogen into the active protease plasmin. Astrup and his co-workers (Astrup & Permin, 1947; Albrechtsen, 1957), who made a detailed study of its distribution in various tissues, used thiocyanate extracts and the fibrin-plate-lysis method as a means of estimating quantitatively the activator content in various human and animal tissues. Tagnon & Palade (1950) and Lewis & Ferguson (1950) made the initial study on the subcellular distribution of the activator and its particulate nature by using 0.9% sodium chloride solution for preparing tissue homogenates. Again a fibrin-clot-lysis method was used, but no further biochemical characterization was attempted.

As tissue homogenization and subcellular fractionation techniques have been improved since the above studies were made, and especially since de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) initiated the lysosome concept, it was considered proper to study the subcellular origin and biochemical nature of the plasminogen activator from various tissues and animal species. The present paper describes the distribution of the activator in subcellular fractions from rabbit kidney and the presence in similar fractions of a latent proteolytic enzyme whose activity is enhanced in the presence of Triton X-100 at pH 7.4.

A preliminary communication of part of this work has already been published (Lack & Ali, 1964).

MATERIALS AND METHODS

Preparation of tissue fractions. One-month-old New Zealand red rabbits (1 kg.) were killed by a blow on the neck, and the kidneys were removed, dissected free of fat and capsule, weighed (usually 5 g.), cut up into four pieces and washed three times with chilled 0.25 M-sucrose. The pieces were cut up into smaller pieces with scissors, introduced into a Potter-type homogenizer (smooth-glass-walled tube and Teflon pestle) and homogenized at 4° and 5000 rev./min. for 2 min. (five up-and-down strokes) in sufficient sucrose to make a 10% (w/v) suspension. The homogenate was passed through muslin to remove gross unhomogenized pieces of tissue. After removal of a sample for enzymic estimations the homogenate was subjected to differential centrifugation in an MSE automatic Super-Speed 50 centrifuge at 4° in an angle rotor (no. 2408) (maximum radius 3.074 in.). The following subcellular fractions were prepared, by using the method described by de Duve & Bethet (1954) and the nomenclature adopted by de Duve *et al.* (1955) and Dingle (1961) for rat liver and by Weissmann & Thomas (1962) for subcellular fractions from rabbit livers: (1) nuclear fraction (containing nuclei and cell debris), 800 g for 5 min.; (2) mitochondrial fraction, 5000 g for 10 min.; (3) lysosome-rich fraction, 15000 g for 20 min.; (4) microsomal fraction, 140000 g for 60 min.; (5) supernatant.

Each subcellular fraction was washed once with 10 ml. of 0.25 M-sucrose and recentrifuged at the same speed, the washing fluid being added to the previous supernatant fluid. The washed subcellular fraction was then dispersed in 0.25 M-sucrose to give a final concentration of 20% (w/v),

distributed into portions (6ml.) and retained at -10° . Experiments refer to these samples that were thawed once before use except in instances where freshly prepared lysosome-rich fractions were used. Enzyme estimations of each fraction were carried out in the presence of 0.1% Triton X-100 (Charles Lennig and Co. Ltd., London, W.C. 1). The activities of fractions are expressed: (1) as the amount (mg.) of phosphorus or aromatic amino acids liberated from the substrate/vol. of fraction equivalent to the wet weight of the original tissue; (2) as a percentage of the total recovered activity; (3) as relative specific activity, i.e. as (2) divided by the protein content of the fraction expressed as a percentage of the total recovered protein.

Density-gradient centrifugation. A discontinuous sucrose gradient was used by layering successively 6ml. each of 4, 3, 2 and 1 molal sucrose carefully with a pipette into centrifuge tubes (35ml. total volume) held upright with a clamp. Tubes were then allowed to equilibrate at 4° for 1 hr. A mixture of lysosome-rich and microsome fractions, i.e. the sediment after centrifuging the supernatant from the 5000g centrifuging at 140000g [4ml. of 40% (w/v) suspension in sucrose] freshly prepared the same day was carefully layered over the sucrose gradient and centrifuged at 64000g in an MSE automatic Super-Speed 50 centrifuge at 4° for 1 hr. in a swing-out rotor (no. 2417) (maximum radius 5.125 in.).

The following procedure was adopted for fractionation of the layers after centrifugation (H. R. V. Arnstein, personal communication). A thin metal tube (syringe needle) transversing the centre of a rubber bung was carefully introduced into the centrifuge tube so that the end of the tube just reached its bottom when the rubber bung fitted into its mouth. Samples (1ml.) were then withdrawn with an automatic pipetting unit (Arnold R. Horwell Ltd., London, N.W. 2) into matched tubes so that the turbidity produced by the particles could be determined directly in a portable EEL colorimeter (green filter OGR1). To obtain sufficient of the various enzymes for estimation individual fractions from three different centrifuge tubes (from the same centrifugation) were pooled together and suitably diluted, since it was found that the fractionation was exactly reproducible. Various enzyme estimations were then made on each fraction. To facilitate the expression of different enzymes on the same scale rather than on different individual scales, the results for each tube or fraction were expressed as the percentage of total recovered activity.

Activation of plasminogen. Human plasminogen prepared from human plasma Cohn fraction III (Cohn *et al.* 1946) by the method of Kline (1953) was used. For activation, a portion of plasminogen solution (0.2ml.) equivalent to 30×10^{-3} casein units (Davidson, 1960) of plasmin activity was incubated with 0.2ml. of the subcellular fraction in 0.2M-tris-HCl buffer, pH 7.4, in a final volume of 2ml. at 37° for 30 min. Controls for spontaneously activated plasmin (0.2ml. of plasminogen and buffer) were included under similar conditions but showed negligible activity. Hydrolysis of casein was then measured as described below.

Protease assay. The proteolytic activity of the subcellular fractions as well as plasmin activated by them was determined by the following modification of the caseinolytic method of Kunitz (1947). Cellular fraction (0.2ml.) was added to enough 0.2M-tris-HCl buffer, pH 7.4, to bring the final volume to 2ml. Triton X-100 [0.1ml. of 2%

(v/v) solution], sodium deoxycholate, ϵ -aminohexanoic acid (ϵ -aminocaproic acid) (L. Light and Co. Ltd., Colnbrook, Bucks.) or soya-bean trypsin inhibitor (Northrop, Kunitz & Herriott, 1948) was included in the final volume in the amounts indicated in the text. To this mixture of cellular fraction in 2ml. of buffer or to the 2ml. of activated plasminogen (see above) 1 ml. of 4% (w/v) casein in tris-HCl buffer, pH 7.4, was added, mixed and incubated at 37° for 1 hr. Then 3ml. of 10% (w/v) trichloroacetic acid was added to stop the casein digestion. Appropriate controls were prepared to which trichloroacetic acid was added immediately after the addition of casein. The precipitated protein was removed by filtration through Whatman no. 3 filter paper. Extinction values were determined on the filtrates in a Unicam SP.500 spectrophotometer at $280m\mu$ in cells of 1 cm. light-path. The difference in extinction values between the control and test samples represents the extent of proteolytic or activator activity in 1 hr. by 0.2ml. of cell fraction with or without plasminogen under the conditions of the assay. To measure the fibrinogenolytic activity instead of the caseinolytic activity, in one experiment 1ml. of 4% (w/v) bovine fibrinogen in tris-HCl buffer, pH 7.4, was substituted for casein under exactly the same conditions.

Activation and protease assay at different pH values. To assay the proteolytic activity of the lysosome-rich fraction at different pH values, 0.2ml. of 20% (w/v) suspension of the fraction under investigation was made up to 2ml. with the following buffers at the appropriate pH: 0.2M-acetate buffer in the range pH 4.6-6.0; 0.2M-tris-HCl buffer in the range pH 6.4-10; 0.2M-glycine-NaOH buffer at pH 10.9. Triton X-100 [0.1ml. of 2% (v/v) solution] and plasminogen (0.2ml.; 30×10^{-3} units) were included whenever mentioned. In this assay 1ml. of 4% (w/v) casein, made up in distilled water was used, so that the digestion could take place at the pH value selected. The rest of the procedure was exactly the same as for the protease assay described above.

Activation of plasminogen at different pH values. Lysosome-rich fraction (0.2ml.) was mixed with 0.5ml. of 0.1M buffer of different pH values in the range mentioned above. Triton X-100 [0.1ml. of 2% (v/v) solution] and human plasminogen (0.2ml.; 38×10^{-3} units) were added and the mixtures incubated at 37° for 15 min. After activation of plasminogen, 1ml. of 0.2M-tris-HCl buffer, pH 7.4, was added, followed by 1ml. of 4% (w/v) casein solution in 0.2M-tris-HCl buffer, pH 7.4, and the digestion was allowed to take place at 37° for 15 min. Then 3ml. of 10% (w/v) trichloroacetic acid was added and the usual procedure followed. Activation is represented by the difference between digestion of casein by plasmin and lysosome-rich fraction, and that by the lysosomal protease on its own.

Differentiation and expression of proteolytic and activator activities. In all the above estimation procedures for protease and activator activities, casein is being hydrolysed simultaneously by at least three proteolytic enzymes, and the following procedures have been used to differentiate between their individual activities: *A*, spontaneous plasmin was estimated by incubating plasminogen alone with casein; *B*, the tissue protease was estimated by incubating the tissue fraction only with casein; *C*, the combined activity of tissue protease and plasminogen activated by tissue fraction was estimated by incubating the tissue fraction and plasminogen with casein. Thus *A* gives the extent of

hydrolysis by any spontaneously formed plasmin, which is known to be a minor contaminant of all plasminogen preparations, and *B* gives the extent of hydrolysis by the tissue protease. The difference $C - (A + B)$ gives the extent of the plasmin formed by activation of plasminogen by the tissue activator, and it is this difference in the extinction that is referred to below as tissue activator activity. The amount of total activity in individual subcellular fractions in Table 1 was estimated by converting the difference in extinction at 280 m μ between the control and test samples into μ g. of acid-soluble tyrosine released by reading against a standard tyrosine curve. It was found that E_{280} 0.115 was equivalent to 100 μ g. of tyrosine under the conditions of the test procedure. This method was adopted for expression of results in only Table 1, as it was preferred to expressing the total activity either in terms of *E*, or introducing an arbitrary unit at this stage of the initial studies on crude enzyme fractions. The results in Table 1 for proteolytic enzymes and activator of plasminogen are thus an expression of the absorption by all the aromatic residues in the filtrate including tyrosine, tryptophan and phenylalanine.

Distinction between the effects of Triton X-100 on soluble neutral protease, plasminogen activation and plasmin. The effect of Triton X-100 on the tissue soluble protease and on the different stages in the activation of plasminogen was investigated by the following procedure. Triton X-100 [0.1 ml. of 2% (v/v) solution] was added in the assay system, wherever mentioned, either before the inclusion of the lysosome-rich fraction and plasminogen or added after the activation of plasminogen by the lysosomal activator and after the addition of the substrate casein. Rabbit-kidney lysosome-rich fraction [3.5 ml. of 40% (w/v) suspension in sucrose] was either diluted to 7 ml. with 0.2 M-tris-HCl buffer, pH 7.4, and assayed directly or was diluted and solubilized by the following two methods: (a) 3.5 ml. of lysosome-rich fraction was diluted with 2 ml. of water and then adjusted to pH 10.5 on a pH-meter (glass electrode) by adding 0.1 N-NaOH; the volume was then made up to 7 ml. with water; (b) 3.5 ml. of the lysosome-rich fraction was diluted to 7 ml. with tris-HCl buffer, pH 7.4, and blended in an MSE steel-blade homogenizer at 10000 rev./min. for 5 min. Both (a) and (b) were then centrifuged at 15000 g for 20 min., and the supernatants decanted and used as a source of tissue soluble protease and activator in the presence and absence of Triton X-100 as described above. Streptokinase [40 units (Dornokinase, as supplied by Burroughs Wellcome and Co.), the activity (Christensen, 1949) being taken to be that indicated on the vials] was used as a source of a pure activator of plasminogen to compare the effect of Triton X-100 on different stages in the formation and action of plasmin.

Determination of cathepsin at pH 5.6. The same procedure was followed as for the estimation of protease activity at pH 7.4 except that the cellular fractions were made up to 2 ml. in 0.2 M-acetate buffer, pH 5.6, and 1 ml. of 4% (w/v) casein made up in distilled water was added. The assay was carried out in the presence of 0.1% Triton X-100.

Determination of phosphatase activity. The acid-phosphatase activity of the subcellular fractions was determined by the method of Gianetto & de Duve (1955) by using sodium β -glycerophosphate as substrate at pH 5.0 in the presence of 0.1% Triton X-100. Glucose 6-phosphatase was estimated by the method described by de Duve *et al.*

(1955) with the appropriate pure substrate in 0.2 M-tris-HCl buffer, pH 6.5. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925) and the results are expressed as the amount of phosphorus liberated in 30 min. of incubation under the conditions described for the above methods.

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1951) was employed, with reconstituted freeze-dried serum (Glaxo Laboratories Ltd., Greenford, Middlesex) of known protein content as standard.

EXPERIMENTAL AND RESULTS

Intracellular distribution of enzymic activities. Table 1 shows the intracellular distribution of the enzymic activities of the five enzymes assayed, namely acid phosphatase at pH 5.0, cathepsin at pH 5.6, protease at pH 7.4, activator of plasminogen at pH 7.4 and glucose 6-phosphatase at pH 6.5. A high proportion of the acid-phosphatase activity consistently sedimented under the conditions described by de Duve *et al.* (1955) and de Duve (1959), and hence this fraction has been termed the lysosome-rich fraction; it always displayed a high percentage of activator activity. Substantial amount of activator activity was also present in the microsome fraction but this was consistently less than that in the lysosome-rich fraction. The nuclear fraction, the mitochondria and the supernatant showed very little activator activity, indicating that this activity was confined to the lysosomes and microsomes, although predominating in the former. The relative specific activity for activator was almost the same in the lysosome and microsome fractions as the latter had a lower percentage of total recovered protein. The cathepsin at pH 5.6 showed an equal distribution in the lysosome and microsome fractions and a higher relative specific activity in the latter. The protease at pH 7.4 seems, however, to be microsomal, as is the glucose 6-phosphatase, as they both showed a high percentage distribution and higher relative specific activity in this fraction when compared with other fractions.

The lysosome-rich fraction contained substantial glucose 6-phosphatase activity, and more than 20% of the acid-phosphatase activity was present in the microsome fraction. For this reason density-gradient centrifugation was attempted to elucidate and confirm the origin of the activator of plasminogen as being in a particular group of subcellular particles. The sedimentation peak for activator activity again coincided with the peak for acid-phosphatase activity (Fig. 1). The sharp activator-activity peak showed a hump that also coincided with the second smaller peak of acid-phosphatase activity at fraction 16. The third smaller peak of activator activity in fraction 22 is probably associated with the neutral-protease activity (see

Table 1. *Intracellular distribution of enzymic activities*

Fraction	Acid phosphatase			Cathepsin			Protease at pH 7.4			Activator at pH 7.4			Glucose 6-phosphatase		
	Total protein (mg.)	Total activity (mg. of phos- phorus)	Relative specific activity	Total activity (mg. of tyrosine)	Relative specific activity	Total activity (mg. of tyrosine)	Relative specific activity	Total activity (mg. of tyrosine)	Relative specific activity	Total activity (mg. of tyrosine)	Relative specific activity	Total activity (mg. of phos- phorus)	Relative specific activity	Total activity (mg. of phos- phorus)	
Nuclear	22.5 (5.0)	0.13 (3.8)	0.75	1.41 (4.0)	0.79	2.94 (7.6)	1.51	0.0 (0.0)	0.00	0.14 (3.5)	0.69				
Mitochondrial	51.0 (11.5)	0.36 (10.6)	0.92	4.56 (13.1)	1.14	2.18 (5.7)	0.49	1.58 (1.4)	0.12	0.35 (8.8)	0.08				
Lysosome-rich	91.0 (20.4)	1.48 (43.5)	2.13	11.10 (32.0)	1.57	11.90 (31.0)	1.52	57.30 (51.6)	2.53	1.49 (37.2)	1.82				
Microsomal	71.5 (16.1)	0.79 (23.4)	1.45	10.90 (31.3)	1.94	16.30 (42.4)	2.63	45.10 (40.7)	2.52	1.60 (40.0)	2.49				
Supernatant	210.0 (47.0)	0.64 (18.9)	0.42	6.85 (19.6)	0.41	5.20 (13.5)	0.29	7.04 (6.3)	0.13	0.42 (10.5)	0.22				
Total activity of the homogenate	432.0 mg.	3.70 mg.	92%	35.0 mg.	99%	51.7 mg.	75%	144.0 mg.	78%	4.52 mg.	90%				
Percentage recovery compared with homogenate	103%

Fig. 1. Density-gradient-centrifugation distribution of rabbit-kidney lysosome-microsome fraction [4 ml. of 40% (w/v) suspension in sucrose] layered over a discontinuous sucrose gradient (4 molal to 1 molal). Percentage activity in each fraction was calculated from the total recovered activity or concentration for: ○, activator of plasminogen; ●, acid phosphatase; △, glucose 6-phosphatase; ▲, protease at pH 7.4; ■, protein content; □, turbidity produced by sedimenting subcellular particles in each fraction.

below). The acid phosphatase was associated with a heterogeneous group of particles, whereas the distribution of glucose 6-phosphatase activity was symmetrically dispersed. The peak for glucose 6-phosphatase activity was 3 fractions or 0.525 cm. away from the peak for activator activity. The protease active at pH 7.4 did not move from the position at which the original material in 0.25 M-sucrose was layered over the molal solution at the

top of the sucrose gradient. This seemed anomalous as the protease activity was particulate (microsomal) when it sedimented on differential centrifugation in 0.25M-sucrose but became dissociated only after this particulate lysosome-microsome fraction was layered on a dense sucrose medium during density-gradient centrifugation. This proteolytic activity was usually associated with a small amount of activator activity (fraction 22), and it is known that some proteolytic enzymes, e.g. trypsin, are able to activate plasminogen.

The total recovery of the various enzymes and protein after density-gradient centrifugation as compared with the lysosome-rich fraction layered at the start of the sedimentation was as follows: protein, 87%; acid phosphatase, 91%; glucose 6-phosphatase, 93%; activator of plasminogen, 88%; protease at pH 7.4, 108%.

Fig. 2. Effect of pH on the proteolytic and activator activities. Lysosome-rich fraction [0.2ml. of 20% (w/v) suspension in sucrose] with or without plasminogen (0.2ml.; 20×10^{-3} units) and Triton X-100 [0.1ml. of 2% (v/v) solution] was allowed to digest casein [1ml. of 4% (w/v) solution] at different pH values at 37° for 1 hr. and the increase in E at 280 $m\mu$ determined. The buffers used are given in the text. ●, Lysosome-rich fraction only; ○, lysosome-rich fraction + Triton X-100; △, lysosome-rich fraction + plasminogen; ▲, lysosome-rich fraction + plasminogen + Triton X-100.

Optimum pH for lysosomal enzymic activities. When proteolytic and activator activities of the lysosomal fraction with and without Triton X-100 (0.1%) were plotted against pH, it was observed that in the absence of Triton the proteolytic activity of the lysosome-rich fraction showed a distinct peak only at pH 5.4 (Fig. 2). The same fraction in the presence of human plasminogen showed a similar peak at pH 5.4, but in addition there was another much larger peak due to the activated plasmin in the neutral pH range. When assayed in the presence of 0.1% Triton X-100, however, the protease activity of the lysosomal fraction in the absence of plasminogen showed activity under neutral conditions in addition to the peak at pH 5.4. The curve for the activity of the lysosomal fraction plus plasminogen in the presence of Triton was very similar to that in the absence of Triton except for a proportionate enhancement in the activity at all the values of pH tested. The release of neutral-proteolytic activity from the lysosomal fraction by Triton was shown in several different experiments.

Action of Triton and heat on lysosomal enzymes. The following experiments were undertaken to study the nature and characteristics of the tissue activator and protease present in the lysosome-rich fraction to see if they had enzymic properties and whether these activities could be ascribed to the same or different enzymes.

When freshly prepared lysosome-rich fraction [2ml. of 20% (w/v) suspension in 0.25M-sucrose] in the presence of 0.2ml. of 2% (v/v) Triton X-100 and 1.8ml. of 0.2M-tris-hydrochloric acid buffer, pH 7.4, was centrifuged at 15000g for 20min., 61.3% of the original activator activity and 80.5% of the proteolytic activity were released into the supernatant fluid (Table 2). When the lysosome fraction was diluted with 0.25M-sucrose without the addition of Triton (as a control) and centrifuged, the supernatant displayed only 9.8% of the original activator activity and 33% of the neutral-proteolytic activity. This indicated that most of the activator and protease activities was latent and bound to the lysosomes and required the surface-active agent Triton X-100 to facilitate the release from the particle membranes. An instantaneous clearing of the opalescent solution was noted on the addition of Triton, and there was an immediate release of latent enzymic activities from the subcellular fraction.

When the lysosome-rich fraction [40% (w/v) suspension in sucrose] was heated at 80° for 15min. and then assayed at pH 7.4, 87% of the original protease activity and 90% of the activator activity were lost owing to the heat denaturation of the proteins. When heated at 56° for 30min. the lysosome-rich fraction lost only 40% of both the

Table 2. *Effect of Triton X-100 on the release of enzymes from lysosome-rich fraction*

Freshly prepared rabbit-kidney lysosome-rich fraction [2 ml. of 20% (w/v) suspension in sucrose] was diluted either with 0.2 ml. of 2% (v/v) Triton X-100 in 1.8 ml. of 0.2 M-tris-HCl buffer, pH 7.4, or with 2 ml. of 0.25 M-sucrose and centrifuged at 15000g for 20 min. The supernatants were decanted off and the respective sediments were suspended in Triton-buffer or in sucrose. Triton was subsequently added to the supernatant from the sucrose-treated fraction and to the resuspended sediments so that all diluents had the same concentration of Triton X-100. A sample (0.6 ml.) of these diluents was used to determine the protease and activator activities with 50×10^{-3} units of plasminogen, as described in the text. Duplicates of lysosome-rich fraction without centrifugation were included to obtain total activity in the original tissue fraction.

	<i>E</i> ₂₈₀ after casein hydrolysis	
	Protease activity	Activator activity
Lysosome-rich fraction (2 ml.)+sucrose (2.2 ml.)	0.037	0.157
Lysosome-rich fraction (2 ml.)+tris buffer (2 ml.)+ Triton (0.2 ml.)	0.072	0.203
Lysosome-rich fraction (2 ml.)+sucrose (2 ml.): centrifuged		
Supernatant+Triton (0.2 ml.)	0.024	0.020
Sediment suspension+Triton (0.2 ml.)	0.070	0.204
Lysosome-rich fraction (2 ml.)+tris buffer (1.8 ml.)+ Triton (0.2 ml.): centrifuged		
Supernatant+sucrose (0.2 ml.)	0.058	0.124
Sediment suspension+Triton (0.2 ml.)	0.019	0.099

proteolytic and activator activities at pH 7.4. Although this experiment suggested the possibility of the two activities being due to enzymes, it was not possible to distinguish between the two activities by heat treatment.

Effect of lysosome fraction and casein concentrations. The following experiments were done to substantiate the enzymic nature of the enhancement of proteolytic and activator activities of the lysosome-rich fraction in the presence of Triton X-100 and to determine the optimum conditions for enzyme action. When different amounts of the lysosome-rich fraction (from 0.01 to 0.8 ml. of a solution containing 3 mg. of protein/ml.) were incubated in the presence of Triton with casein for 1 hr. with and without the addition of human plasminogen (38×10^{-3} units) the results expressed in Fig. 3 were obtained. The values for activator are derived by taking the difference in extinction obtained by incubating the tissue fraction alone (protease) and by incubating the tissue fraction in the presence of plasminogen (protease plus activated plasminogen). Again, it was not possible to distinguish whether the two activities originate from the same enzyme or not. Fig. 3 also shows the difficulty of working with two proteolytic enzymes hydrolysing the substrate simultaneously, and this may be one of the reasons for a slight disproportionality between enzyme activity and enzyme concentration. Other contributory factors may be due to the fact that a crude tissue fraction was used, or that a shorter incubation time (initial velocity) was not employed. Proteinases acting on proteins have been shown repeatedly to give non-linear enzyme concentration curves, usually showing a deceleration (Dixon & Webb, 1964).

Fig. 3. Effect of lysosome-rich fraction concentration on protease and activator activities. Lysosome-rich fraction [0.2 ml. of 20% (w/v) suspension in sucrose] was incubated with and without plasminogen (0.2 ml.; 38×10^{-3} units) in the presence of Triton X-100 with casein for 1 hr. at 37° and the increase in *E* at 280 mμ determined. ○, Lysosome-rich fraction only; △, lysosome-rich fraction+plasminogen; ●, difference between the above two values, representing activator activity.

Fig. 4. Effect of substrate concentration on protease and activator activities. Lysosome-rich fraction [0.2 ml. of 40% (w/v) suspension in sucrose] was incubated with and without plasminogen (0.2 ml.; 38×10^{-3} units) in the presence of Triton X-100 with various amounts of casein substrate for 1 hr. at 37° and the increase in E at $280 m\mu$ determined. $\circ$, Lysosome-rich fraction only; Δ , lysosome-rich fraction + plasminogen; $\bullet$, difference between the above two values, representing activator activity.

Fig. 5. Effect of time on casein hydrolysis. Lysosome-rich fraction [0.2 ml. of 40% (w/v) suspension in sucrose] was incubated with and without plasminogen (0.2 ml.; 38×10^{-3} units) in the presence of Triton X-100 and casein for various times and the increase in E at $280 m\mu$ determined. $\circ$, Lysosome-rich fraction only; Δ , lysosome-rich fraction + plasminogen; $\bullet$, difference between the above two values, representing activator activity.

Fig. 4 shows the effect of varying the substrate concentration (from 4 to 80 mg. of casein) on the acid-soluble aromatic amino acid residues released by the lysosomal protease [0.2 ml. of 40% (w/v) suspension] and 38×10^{-3} units of plasminogen activated by it. It was possible in this experiment to differentiate between the rate of hydrolysis by the tissue protease and plasminogen activated by the tissue activator. In fact an excess of casein substrate seemed to act as an inhibitor to the lysosomal protease at pH 7.4.

Effect of time on the activation of plasminogen and of casein digestion by the lysosomal protease. When plasminogen (0.2 ml.; 38×10^{-3} units) was incubated with the lysosome-rich fraction [20% (w/v) suspension in sucrose] for various times (from 5 min. to 2 hr.) for activation and then allowed to digest casein for 1 hr., it was found that more than 90% of the activation took place within 5 min.

When a similar lysosome-rich fraction and plasminogen activated by it for 30 min. at 37° were incubated with casein at 37° for various times from 2 min. to 2 hr., the results shown in Fig. 5 were obtained. The rate of hydrolysis by the protease seemed to fall off more rapidly than that for plasminogen activated by tissue activator. Figs. 5 and 3 show that a shorter incubation period than that employed by Kunitz (1947) would have to be used to measure the initial velocities and to enable useful arbitrary units for the enzymes under study to be defined.

Effect of pH on the activator activity. The activation of human plasminogen by the lysosome-rich fraction at different pH values is shown in Fig. 6. Although there is substantial activator activity at pH 7.4, maximum activity seems to be

at pH 9.0. Most of the activator activity is not associated with the cathepsin at pH 5.4, but is in the neutral-alkaline pH range.

Effect of inhibitors on proteolytic and activator activity. An attempt was made to distinguish between the proteolytic and the plasminogen-activator activities of the rabbit lysosomal fraction by the use of certain inhibitors. The three inhibitors used, ϵ -aminohexanoic acid (from 0.01 to 0.4 M) (Alkjaersig, Fletcher & Sherry, 1959), arginine (0.1 M) and soya-bean trypsin inhibitor (0.1%) in

the final concentrations mentioned showed almost 100% inhibition of activation of plasminogen by the lysosomal activator (Table 3). These compounds in the same concentrations did not inhibit the acid cathepsin (pH 5.6) or the lysosomal neutral protease. Only arginine showed certain inhibition of the protease activity (34.6%) at pH 7.4. These observations suggest that the proteolytic and activator activities may be properties of two different enzymes.

Effect of release agents on the proteolytic and activator activities. Table 4 summarizes the effect of Triton X-100 (final concn. 0.1%) and sodium deoxycholate (final concn. 0.1%) on the enhancement of the proteolytic and activator activities of lysosomal enzymes. Besides comparing the effect of the two surface-active agents this experiment also differentiates between the tissue activator and tissue protease in the presence of ϵ -aminohexanoic acid. Since all the samples of the lysosomal fraction were frozen and thawed once, this was not a strict test for the latency of the enzymes as any limiting lipoprotein membrane would be disrupted by the freezing and thawing and also by the hypo-osmotic conditions of the assay. The proteolytic activity of the fraction was increased twofold at pH 5.6 and nearly fourfold at pH 7.4 in the presence of Triton. There was nearly a twofold rise in the neutral-protease activity in the presence of deoxycholate. It thus seems possible that surface-active agents enhance proteolytic activity of the lysosomal fraction in some other way besides releasing enzymes from the limiting membranes of fresh subcellular particles, as was shown in a previous experiment (Table 2). The activator activity was slightly enhanced in the presence of Triton X-100, but deoxycholate seemed to inhibit either the activation or the proteolytic activity of the activated plasmin, as casein hydrolysis was inhibited by

Fig. 6. Effect of pH on the activation of plasminogen by lysosome-rich fraction. Lysosome-rich fraction [0.2 ml. of 40% (w/v) suspension in sucrose] was incubated with and without the addition of plasminogen (0.2 ml.; 38×10^{-3} units) in the presence of Triton X-100 at 37° for 15 min. in different buffers, neutralized with tris-HCl buffer, pH 7.4, and allowed to digest casein for another 15 min. Activation is represented by the difference between digestion of casein by plasmin and lysosome-rich fraction, and that by the lysosome-rich fraction on its own.

Table 3. *Effect of inhibitors on proteolytic and activator activities*

Rabbit-kidney lysosome-rich fraction [0.2 ml. of 20% (w/v) suspension in sucrose] and human plasminogen (0.2 ml.; 20×10^{-3} units) were incubated in the presence of Triton X-100 [0.1 ml. of 2% (v/v) solution] with casein at 37° for 1 hr. and the difference in extinction at 280 m μ was measured, as described in the text, with one of the inhibitors indicated.

	Percentage inhibition of casein hydrolysis						Soya-bean trypsin inhibitor (0.1)
	ϵ -Aminohexanoic acid					Arginine (0.1 M)	
	(0.4 M)	(0.2 M)	(0.1 M)	(0.05 M)	(0.01 M)		
Lysosomal cathepsin at pH 5.6	0	0	0	3	0	8.8	0.1
Lysosomal protease at pH 7.4	0	0	0	0	0	34.6	2.3
Activation of plasminogen by lysosomal activator at pH 7.4	100	100	93	82.7	68.1	100	100

Table 4. *Effect of surface-active agents on proteolytic and activator activities*

Rabbit-kidney lysosome-rich fraction [0.2 ml. of 40% (w/v) suspension in sucrose] and human plasminogen (0.2 ml.; 38×10^{-3} units) were used in the protease assay, as described in the text.

	<i>E</i> ₂₈₀ after casein hydrolysis at 37° for 1 hr.		
	Without releasing agent	Triton X-100 (0.1%)	Deoxycholate (0.1%)
Lysosomal cathepsin at pH 5.6	0.126	0.216	—
Lysosomal protease at pH 7.4	0.057	0.239	0.109
Lysosomal protease + ϵ -aminohexanoic acid (0.1 M) at pH 7.4	0.065	0.233	0.096
Lysosomal protease + plasminogen at pH 7.4	0.508	0.753	0.203
Lysosomal protease + plasminogen + ϵ -aminohexanoic acid (0.1 M)	0.147	0.382	0.230

Table 5. *Comparison of caseinolytic and fibrinogenolytic activities*

Rabbit-kidney lysosome-rich fraction [0.2 ml. of 40% (w/v) suspension in sucrose] and human plasminogen (0.2 ml.; 38×10^{-3} units) activated by it were assayed in the presence of Triton X-100 [0.1 ml. of 2% (v/v) solution], as described in the text.

	<i>E</i> ₂₈₀ after protein hydrolysis at 37° for 1 hr.		
	Fibrinogen (4% w/v)	Fibrinogen (4%, w/v) + ϵ -aminohexanoic acid (0.1 M)	Casein (4%, w/v)
Lysosomal protease at pH 7.4	0.087	0.074	0.270
Lysosomal protease + plasminogen at pH 7.4	0.384	0.127	0.823

about 73% in its presence as compared with Triton X-100.

ϵ -Aminohexanoic acid again seemed to inhibit the activator without inhibiting the neutral protease. From this experiment it appeared that the inhibitory effect of ϵ -aminohexanoic acid was less than 100%, and it is possible that it inhibited tissue activator completely but was not able to inhibit the tissue protease, which may also possess a secondary ability to activate plasminogen (as mentioned above).

Comparison of caseinolytic and fibrinogenolytic activities. The effect of the lysosomal protease on fibrinogen as substrate was studied. The results are summarized in Table 5. It was clear that bovine fibrinogen was one of the substances capable of hydrolysis by the lysosomal protease on its own at pH 7.4, as this activity was not inhibited even in the presence of 0.1 M- ϵ -aminohexanoic acid, as this would have inhibited the activation of any plasminogen contaminating the fibrinogen preparation. The effect of further addition of human plasminogen (0.2 ml.; 38×10^{-3} units) was again nullified by ϵ -aminohexanoic acid, but not the original protease activity of the lysosomal fraction. A comparison with casein showed that much more trichloroacetic acid-soluble material

was released from casein than from fibrinogen by both the lysosomal protease and by activated plasminogen. This indicates that at the concentration tested (4%, w/v) casein is a more effectively hydrolysable substrate for both these proteolytic enzymes than is fibrinogen under the conditions of the assay method used.

Distinction between the effects of Triton X-100 on soluble neutral protease, plasminogen activation and plasmin. Rabbit-kidney lysosome-rich fraction was assayed, either directly after diluting with buffer or after diluting and solubilizing by either of the two methods described in the Materials and Methods section, to determine the effect of Triton X-100 on the soluble protease, on plasminogen, on the activation of plasminogen by tissue soluble activator and on active plasmin. Table 6 summarizes the results obtained. Triton X-100 had virtually no effect on plasminogen or on the small amount of plasmin that is known to contaminate all plasminogen preparations. Neither did it have any effect on streptokinase, or on the process of activation of plasminogen by streptokinase, or on plasmin formed, as there was not much difference in the values obtained with and without Triton, or with the addition of Triton after the addition of casein. The neutral-protease activity, on the other hand,

Table 6. *Distinction between the effects of Triton X-100 on soluble neutral protease, plasminogen activation and plasmin*

Rabbit-kidney lysosome-rich fraction [3.5 ml. of 40% (w/v) suspension in sucrose] was either assayed directly after diluting to 7 ml. with tris-HCl buffer, pH 7.4, or was diluted and solubilized (see the text), centrifuged and the supernatant used for assaying the enzymes. Human plasminogen (50×10^{-3} units) and 0.4 ml. of the diluted lysosome fraction or supernatant solution were used in this experiment for protease and activator assays.

	Without Triton	In the presence of 1% (v/v) Triton X-100	
		Added before activation	Added after addition of casein
Plasminogen (spontaneous plasmin contamination)	0.031	0.027	—
Streptokinase (40 units)	0.005	0.006	—
Plasminogen + streptokinase (plasmin)	0.477	0.468	0.449
Lysosome-rich fraction (protease)	0.065	0.352	—
Lysosome fraction + plasminogen (plasmin)	0.414	0.352	0.327
(a) pH 10.5 supernatant (protease)	0.056	0.341	—
pH 10.5 supernatant + plasminogen (plasmin)	0.461	0.322	0.306
(b) Supernatant after blender treatment (protease)	0.031	0.274	—
Supernatant after blender treatment + plasminogen (plasmin)	0.370	0.287	0.271

was enhanced almost sixfold when either the whole lysosome fraction or the supernatant at pH 10.5 was used, and almost ninefold when the supernatant from blender treatment was used, indicating that Triton is increasing the proteolytic activity of the soluble enzyme in addition to its ability to release this enzyme from fresh lysosomes (Table 2). The effects of Triton X-100 on the activation of plasminogen by tissue activator or on plasmin formed by tissue activator are difficult to interpret. There was certainly no enhancement by Triton of the activator activity or of the plasmin formed; in fact it appeared from the values obtained as if these activities were being inhibited by Triton. This inhibitory effect of Triton may be an artifact, for it is possible that in its presence the enhanced soluble protease competes with plasmin (formed by the action of tissue activator) for the substrate casein. This combined proteolytic activity in the presence of Triton would leave plasmin with comparatively less substrate and this may explain the lower values for the activation of plasminogen and for plasmin in Table 6.

This experiment also showed that a greater proportion of the total lysosomal activity could be made soluble by diluting the tissue fraction and lowering the H^+ ion concentration to pH 10.5 than by the blender treatment at pH 7.4. There was a visible clearing of the particulate fraction at pH 10.5 and much less sediment was obtained after centrifugation than with the blender treatment.

DISCUSSION

The subcellular fractionation of rat lung in 30% sucrose by Tagnon & Palade (1950) and of various

dog tissues in iso-osmotic saline by Lewis & Ferguson (1950) had led to the conclusion that the activator activity resides equally in the microsomal and the mitochondrial fraction. Our studies indicate that the activator probably originates or is stored in the lysosomes, which have sedimentation properties in between the mitochondria and microsomes, and this would be consistent with results of the above workers. With casein as substrate we did not find any inhibitor of plasmin in the supernatant fraction, as was suggested by Lewis & Ferguson (1950), who used the fibrin-digestion method, but in fact some residual activator activity was present in this fraction. Such an anomaly can best be explained by the recent finding that the fibrin-digestion method is complicated by the presence in mammalian tissues of a fibrin-stabilizing factor (as distinct from a plasmin inhibitor), which appears to make the stabilized fibrin resistant to plasmin action (Tyler & Lack, 1964). Differential centrifugation showed a high percentage of the activator to be present in the lysosome-rich fraction and this was further confirmed by density-gradient centrifugation. The distribution of acid-phosphatase activity after density-gradient centrifugation was not simple and is consistent with more recent concepts of the heterogeneity of the lysosome particles in cells (de Duve, 1963).

In the absence of releasing agents and plasminogen the lysosome-rich fraction displays substantial proteolytic activity only at pH 5.4. At neutral pH, owing to its activator content, this fraction can convert plasminogen into plasmin. In the presence of releasing agents such as Triton X-100 and deoxycholate the lysosome-rich fraction with-

out plasminogen showed significant proteolytic activity over a broad neutral pH range. This proteolytic activity at neutral pH as a result of the enhancement of a tissue protease by Triton X-100 is distributed in the cell fractions in the same manner as the glucose 6-phosphatase and is hence likely to be microsomal in origin. Although the presence of proteolytic enzymes in tissue homogenates active at neutral pH has already been reported (Hanson, Blech, Hermann & Kleine, 1959; Fruton, 1960), it has now been shown that most of this activity is latent and requires specific releasing agents to attain maximum activity.

The enzymic nature of the tissue activator of plasminogen and of the neutral protease enhanced in the presence of Triton X-100 was substantiated and confirmed by various experiments, including loss of activity on heating or in the presence of specific inhibitors, dependence of the activity on optimum pH, on the enzyme and substrate concentration and on time of incubation with the substrate. It was also found that the activator and tissue protease activities were latent and bound to the lysosomes in the presence of 0.25M-sucrose, and that these activities were released by Triton X-100, possibly by the disruption of the limiting lipoprotein membranes (de Duve, 1959). Triton X-100 had no enhancing effect on tissue soluble activator, on activation of plasminogen, or on active plasmin, but it increased the activity of tissue soluble protease, possibly by removal of lipid inhibitors or by prevention of adsorption of the tissue protease on to surfaces including the glass walls of the test tubes used.

Deoxycholate increased twofold the original proteolytic activity of the lysosomal fraction but was much less effective in this respect than Triton X-100. The inhibition of plasmin activity by deoxycholate is of interest because high concentrations of cholesterol in blood inhibit fibrinolysis *in vivo* (Kwaan & McFadzean, 1957). In general, only inhibitors of plasminogen activation have been found and none of lysosomal proteolytic activity, which may suggest that those two activities are due to two different enzymes. Other experimental evidence, and especially the different distribution on density-gradient centrifugation, confirms that the activator activity and tissue proteolytic activity are separable. It is possible, however, that the tissue protease may have a secondary property of activating plasminogen similar to the action of trypsin, but this is distinguishable from the bulk of the activator activity due to a specific tissue activator.

Activator of plasminogen showed a broad peak of maximum activity at pH 9.0, similar to that for urokinase activity (Kjeldgaard & Ploug, 1957; Celander & Guest, 1960), and both activators are

inhibited by ϵ -aminohexanoic acid, pointing to their possible similarity. Ladehoff (1960) found activator activity in the kidney, ureter and mucous membrane of the bladder, and human urokinase injected intramuscularly into rabbits has been found (Celander & Guest, 1960) to be passed into urine without losing its antigenic or enzymic activities. Celander & Guest (1960) concluded that endogenous activators, elaborated in various tissues, exist in blood in a neutralized form and are passed through the kidney into the urine. Further, Kaulla (1959) has reported that alterations in the fibrinolytic activity of blood and plasma are related to alterations in urokinase excretion, and he ascribed this finding to a possible systemic origin of urokinase, possibly at sites other than the kidney. Our findings that the activator exists in subcellular particles rather than in the cytoplasmic supernatant fluid indicates that there are specific organelles in kidney that either synthesize or store it, and this is in agreement with the findings of Painter & Charles (1962), who found that a similar activator of plasminogen was elaborated by monkey and dog kidney-cell cultures *in vitro* and extruded into the medium.

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