Purification of Rabbit Kidney Cytokine and a Comparison of its Properties with Human Urokinase

By S. Y. ALI AND LOIS EVANS

*Department of Pathology, Institute of Orthopaedics (University of London),
Royal National Orthopaedic Hospital, Stanmore, Middlesex*

(Received 15 September 1967)

1. The cytokine (tissue activator of plasminogen) content of several mammalian tissues was evaluated by a quantitative casein hydrolysis method. 2. An alkaline (pH 10.5) extraction of cytokine from rabbit kidney lysosome-microsome fraction, followed by chromatography on DEAE-cellulose at pH 7.6 with stepwise or linear increase in concentration of phosphate buffer, gave an 86-fold purification of the enzyme. The purified material was non-proteolytic against casein and heated fibrin and was freeze-dried without significant loss of activity or solubility. 3. Cytokine is a protein with $E_{1cm}^{0.1\%} = 0.87$ at 280 m μ , and does not possess sufficient hexose or sialic acid to be classified as a glycoprotein. It has $S_{20,w}$ 2.9–3.1s and molecular weight 50 000 when measured on a calibrated Sephadex G-100 column. It has an isoelectric point between pH 8 and pH 9, and is maximally active and stable at pH 8.5. It is inactivated by heat at 78°. 4. Cytokine and human urokinase have the same K_m value and are inhibited in a partially competitive manner by ϵ -aminohexanoic acid and aminomethylcyclohexanecarboxylic acid. They are also inhibited by cysteine and arginine, but are unaffected by iodoacetamide and *p*-chloromercuribenzoate. 5. On the basis of this and other evidence it is suggested that rabbit kidney cytokine and human urokinase are similar, if not identical, enzymes.

The activation of the blood proenzyme plasminogen to the active protease plasmin by specific bacterial activators (streptokinase and staphylokinase) and by a urinary activator (urokinase) has been studied extensively. These studies have emphasized the role of plasmin as a fibrinolytic enzyme in thromboembolic and haemorrhagic diseases, and the preplasmin-plasmin-antiplasmin system has been the subject of many recent reviews (Sherry, Fletcher & Alkjaersig, 1959; Von Kaulla, 1963; MacFarlane, 1964; Fearnley, 1965).

Mammalian tissues also contain an activator that converts plasminogen into plasmin, but its study has been hampered by the fact that it appears to be particulate (Astrup & Permin, 1947; Lewis & Ferguson, 1950; Tagnon & Palade, 1950; Lack & Ali, 1964; Astrup, 1966). Astrup and his colleagues made an extensive survey of the distribution of plasminogen activator in tissues from different animals (Astrup & Permin, 1947; Albrechtsen, 1959) and finally attempted a purification of activator from pig heart by extracting it with 2M-potassium thiocyanate (Abe & Astrup, 1960). The only successful purification, however, was achieved by Bachmann, Fletcher, Alkjaersig &

Sherry (1964). Their preparation revealed some heterogeneity as well as residual proteolytic activity, and possessed limited stability and solubility at neutral pH.

An earlier report from our Laboratory showed that over 90% of the activator activity of rabbit kidney was localized in the lysosome-microsome fraction (Ali & Lack, 1965). Substantial amounts of the activator activity could be released from these particulate fractions either by incubation with Triton X-100 or by adjusting to pH 10.5. The present paper describes some experiments on the stability and solubilization of tissue activator from rabbit kidney and its partial purification by column chromatography. The properties of the purified tissue activator were compared with those of human urokinase. To study the purification steps a quantitative caseinolytic method was employed that eliminated the artifacts encountered when a fibrin-lysis method was used.

The purified tissue activator of plasminogen is referred to as cytokine. This term, introduced by Painter & Charles (1962), is consistent with the custom of naming activators of plasminogen so that the first part of the name denotes the origin and the

second part the function of the activator. A preliminary communication describing part of this work has already been published (Ali, 1967). Most of the work has been described in detail elsewhere (Ali, 1966).

MATERIALS AND METHODS

Preparation of lysosome-microsome fraction. One-month-old New Zealand red rabbits (1 kg.) were killed by cervical dislocation. The kidneys were homogenized in 0.25 M-sucrose to give a 10% (w/v) suspension, and subcellular fractions were separated by differential centrifugation (Ali & Lack, 1965). A lysosome-microsome fraction was obtained by centrifuging at 140 000g for 60 min. the supernatant fluid from a 5000g (10 min.) centrifugation of the homogenate. The sediment was resuspended in either 0.25 M-sucrose or water so that the suspension contained lysosomes and microsomes from 0.5 g. of tissue/ml.

Buffers. These were prepared as described by Gomori (1955). Where a range of pH values was required, a universal buffer was prepared as described by Ellis (1961). The concentration of this was 50 mM with respect to each of the four contributory salts.

Streptokinase and urokinase. Dornokinase (Burroughs Wellcome and Co., Beckenham, Kent) was used as a form of pure streptokinase, the activity (Christensen, 1949) being taken as that indicated on the vials. A preparation of standard urokinase, containing 2400 Ploug units (Ploug & Kjeldgaard, 1957), was a gift from Leo Pharmaceutical Products (Copenhagen, Denmark).

Plasminogen. Human plasminogen was prepared from human plasma Cohn fraction III (Cohn *et al.* 1946) by the method of Kline (1953). It was stored in 2.5 M-HCl at -20° . The activity was assayed as described by Davidson (1960) with streptokinase as activator, and is expressed in terms of casein units.

Other chemicals. Aminomethylcyclohexanecarboxylic acid was a generous gift from A. B. Kabi (Stockholm, Sweden); ϵ -aminoheptanoic acid was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). Light white soluble casein was obtained from British Drug Houses Ltd. (Poole, Dorset). Fibrinogen (Cohn fraction I from bovine plasma; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was prepared as needed as a 0.4% solution in borate-saline buffer, pH 7.4 (Palitzsch, 1915).

Determination of cytokinase activity and definition of arbitrary units. A two-stage assay, based on a modification of the caseinolytic method of Kunitz (1947), was used (see Ali & Lack, 1965). The first stage consisted of the activation of plasminogen (50 milliunits) by the cytokinase preparation for 10 min. at 37° in a volume made up to 2 ml. with 0.1 M-tris-HCl buffer, pH 7.4. The second stage consisted of incubation of the above mixture, now containing active plasmin, with 1 ml. of 4% (w/v) casein in tris-HCl buffer, pH 7.4, for 30 min. at 37° . Appropriate control experiments for the determination of any protease activity of the tissue preparation or of any plasmin formed in the plasminogen preparation in the absence of cytokinase were carried out.

Casein hydrolysis was stopped by the addition of 3 ml. of 10% (w/v) trichloroacetic acid and 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 280 m μ . The increase in E_{280} value, after subtraction of the plasmin or protease blank, was expressed in terms of an arbitrary unit of cytokinase activity, defined as the amount of cytokinase that, when it is allowed to activate 50 milliunits (Davidson, 1960) of plasminogen for 10 min. and the resulting plasmin is allowed to digest casein for 30 min. at pH 7.4 and 37° , produces an E_{280} difference of 0.001.

Fibrin plates. For qualitative determinations and for screening fractions for tissue activator activity, bovine fibrin plates were prepared as described by Astrup & Mullertz (1952). One drop (0.02 ml.) of the cytokinase was applied and the plates were incubated at 37° for about 4 hr. Zones of lysis appeared where the fibrin was hydrolysed by the activated plasmin, and the diameter of these was measured by calipers. By taking the average of two diameters at right angles, the area of lysis was calculated.

Determinations of hexose, sialic acid and protein. Total hexose content of cytokinase was determined by the orcinol- H_2SO_4 method for hexoses (Winzler, 1955) with galactose and mannose solutions as standard. Total sialic acid content of cytokinase was determined by the thio-barbituric acid method (Warren, 1959) with synthetic crystalline *N*-acetylneuraminic acid [Sigma (London) Chemical Co., London, S.W. 6] as standard. For the protein determinations, the method of Lowry, Rosebrough, Farr & Randall (1951) was employed, with reconstituted freeze-dried serum (Glaxo Laboratories Ltd., Greenford, Middx.) as standard.

Column chromatography on DEAE-cellulose. Whatman Chromomedia grade DEAE-cellulose powder (DE11), with a nominal capacity of 1.0 m-equiv./g., supplied as a free base, was used. The washing of the DEAE-cellulose, including one washing in 0.2 M- NaH_2PO_4 , and the packing of the column were done exactly as prescribed by Peterson & Sober (1962). The column size was 45 cm. $\times$ 2.5 cm. and the flow rate was 2 ml./min. Fractions (33 ml.) were collected and stored at 4° .

Gel filtration on Sephadex G-100. Sephadex G-100 (particle size 40-120 μ) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Sephadex gel media were prepared in and eluted with 50 mM-tris-HCl buffer, pH 7.4, containing 0.1 M-KCl (Andrews, 1964). The effective column length was 45 cm. and the internal diameter was 2.5 cm. The flow rate was 0.5 ml./min. Cytokinase and urokinase activity was located in the fractions (2.8 ml.) by the fibrinolysis method. The casein hydrolysis assay was then carried out on the active fractions.

Moving-boundary and paper electrophoresis. Free solution electrophoresis was performed in a Tiselius apparatus (model 35; Carl Zeiss, Jena, Germany) at 100v and 6 ma in 0.1 M-acetate buffer, pH 4.63, for 2 hr. at 11° . Paper electrophoresis was performed on Whatman no. 1 paper in a vertical Perspex tank at room temperature as described by Smith (1960). Duplicate samples (0.02-0.04 ml. containing 100 μ g. of protein) of purified cytokinase and of human serum were applied separately to three 36 cm. $\times$ 5 cm. paper strips and subjected to electrophoresis at 300v and 10 ma. Protein was located by staining for 10 min. in 0.2% Ponceau S in 3% (w/v) trichloroacetic acid. An unstained strip of cytokinase was cut transversely into parallel sections (0.5 cm. wide) that were then placed on fibrin plates. After incubation, zones of lysis appeared. The leading edge of the paper section that showed lysis furthest away from the

Table 1. *Cytokinase content of different rabbit organs*

Organs from a young (1 kg.) female rabbit were homogenized in 0.25 M-sucrose and assayed for protease and cytokinase activity with casein as substrate as described in the text. Triton X-100 (0.1%) was included in the assay system to liberate any particle-bound enzymes. Activity is expressed per g. wet. wt.

Tissue	Protease activity (units/g. of tissue)	Cytokinase activity (units/g. of tissue)	Cytokinase specific activity (units/mg. of protein)
Kidney	11100	4750	88
Spleen	7050	3650	79
Lung	5500	3250	70
Brain	2200	2250	63
Liver	7300	3500	57
Uterus	1150	1700	51
Heart	1150	1200	29

Table 2. *Cytokinase content of organs from various animal species*

Rabbit kidneys were taken from rabbits killed in the laboratory; all other organs were from animals killed in the local abattoir. Tissues were homogenized in 0.25 M-sucrose and assayed for cytokinase activity against casein as described in the text. Triton X-100 (0.1%) was included in the assay system to liberate any particle-bound enzymes. Activity is expressed per g. wet wt. The weights of the rabbits are given in parentheses.

Tissue	Cytokinase activity (units/g. of tissue)	Cytokinase specific activity (units/mg. of protein)
Rabbit (0.6 kg.) kidney	10440	117
Rabbit (1 kg.) kidney	5650	75
Rabbit (1.3 kg.) kidney	2800	41
Rabbit (2.5 kg.) kidney	2350	29
Pig kidney	2800	31
Goat kidney	2100	14
Pig heart muscle	1050	7
Calf kidney	660	7
Sheep kidney	200	4
Ox kidney	Nil	Nil

origin was taken as the maximum distance migrated by cytokinase.

Analytical ultracentrifugation. A Spinco model E analytical ultracentrifuge was used. A dialysed cytokinase sample (3000 units/mg.) containing 4.72 mg. of protein in 0.5 ml. was analysed at 20° with a synthetic-boundary (valve-type) cell at a maximum centrifuge speed of 59780 rev./min. (259700 g_{av}). The protein concentration was calculated from the extinction coefficient of cytokinase.

Fig. 1. Effect of various media on the solubilization of cytokinase. Samples of rabbit kidney lysosome-microsome fraction were diluted (1:2) to contain the following: (●) 0.1 M-phosphate buffer, pH 7.6; (○) 0.05 M-universal buffer, pH 10; (×) 0.1 M-pyrophosphate buffer, pH 7.8; (▲) 0.1 M-tris-HCl buffer, pH 7.4; (△) 0.15 M-NaCl in 0.1 M-tris-HCl buffer, pH 7.4; (□) 0.1% Triton X-100 in 0.1 M-tris-HCl buffer, pH 7.4; (■) 50 mM-EDTA in 0.1 M-tris-HCl buffer, pH 7.4; (+) M-potassium thiocyanate. After centrifugation at successively higher speeds, cytokinase in each resuspended sediment and in the final supernatant was determined. The specific activity of cytokinase in the supernatant at each stage was calculated and plotted against centrifugal force on a logarithmic scale.

EXPERIMENTAL AND RESULTS

Distribution of cytokinase activity in animal tissues. Table 1 shows the cytokinase content of various rabbit tissues. Kidney appeared to be the richest source of cytokinase on the basis of total as well as specific activity. All the tissues displayed protease activity against casein, and this was particularly high with kidney and spleen. The total cytokinase activities of kidneys from rabbits of different ages, as indicated by the different weights, are compared in Table 2 with the activities of a variety of other animal tissues.

Comparison of methods for obtaining cytokinase in solution. The effect of various extraction media on the solubilization of cytokinase from rabbit kidney was tested as follows. Samples of a lysosome-microsome suspension in water were diluted individually as indicated in the legend to Fig. 1. They were then centrifuged for 10 min. at successively higher speeds, the sediments at each stage being resuspended in tris-hydrochloric acid buffer, pH 7.4. After the final centrifugation (140000 g for 20 min.) all the sediments and the final supernatant fluids were assayed and the specific activity of cytokinase in each successive supernatant fraction was worked out and plotted against the centrifugal

Fig. 2. Stepwise elution of cytokinase from a DEAE-cellulose column. An alkaline (pH 10.5) extract of a rabbit kidney lysosome-microsome fraction was dialysed against 1 mm-phosphate buffer. The non-diffusible material (19 ml.) was layered on a DEAE-cellulose column and eluted with stepwise increases in the concentration phosphate buffer, pH 7.2. In the final elution step, 0.15 M-phosphate buffer was supplemented with 0.38 M-NaCl. Fractions (33 ml.) were collected and assayed for protein and cytokinase as described in the text. ●, Protein; □, total cytokinase activity; ○, fibrinolytic activity.

Table 3. Purification of cytokinase by column chromatography

Rabbit kidney lysosome-microsome fraction was extracted with 50mm-phosphate buffer, pH 7.6, and the cytokinase extract dialysed overnight against 5mm-phosphate buffer at 4°. The non-diffusible material was layered on a DEAE-cellulose column. Fractions (33 ml.) were collected after elution with 200ml. of each of the following phosphate buffers, pH 7.6: 0.01 M, 0.05 M, 0.1 M, and 0.1 M in 0.75 M-NaCl.

Fraction	Volume (ml.)	Total protein (mg.)	Total activity (units)	Specific activity (units/mg. of protein)	Yield (%)	Purification
Rabbit kidney homogenate	108	1060	54 000	51	100	1
Lysosome-microsome fraction	22	199	40 000	201	74	4
Dialysed supernatant from lysosome-microsome fraction	16	70	11 100	158	20	3
Column fractions 10, 11 and 12	99	6	10 250	1600	19	31
Column fraction 10	33	1	4600	4380	8	86

force. Two phosphate buffers and an alkaline (pH 10) treatment yielded the highest recovery of tissue activator in a soluble form, and there was a rise in specific activity in the supernatant fluid with increasing centrifugal force (Fig. 1).
Fractionation of soluble cytokinase preparations on DEAE-cellulose. The supernatant obtained from centrifuging a lysosome-microsome fraction at pH 10.5 for 10 min. at 20 000g was neutralized to pH 7.2 with n-hydrochloric acid, dialysed against

1 mm-phosphate buffer, pH 7.2, and layered over a DEAE-cellulose column equilibrated with the same buffer. Four major protein peaks were eluted with four stepwise increases in concentration of buffer (Fig. 2). Almost 50% of the cytokinase activity was eluted with the 50 mm-phosphate buffer in a peak that constituted only about 6% of the total protein.
When the lysosome-microsome fraction was extracted with 50 mm-phosphate buffer at pH 7.6

Fig. 3. Linear gradient elution of cytokinase from a DEAE-cellulose column. An alkaline (pH 10.5) extract of a rabbit kidney lysosome-microsome fraction was dialysed against 4 mM-phosphate buffer, pH 7.6, and a sample (12 ml.) was layered on a DEAE-cellulose column equilibrated with the same buffer. The sample was eluted at room temperature (22°) with a linear concentration gradient of phosphate buffer, increasing from 4 mM (300 ml.) to 0.4 M (300 ml.). Fractions (33 ml.) were collected and assayed for protein and cytokinase as described in the text. ●, Protein; □, total cytokinase activity; ○, fibrinolytic activity; △, concn. of phosphate buffer.

and the extract dialysed against 5 mM-phosphate buffer before being layered on a DEAE-cellulose column, essentially the same elution pattern was obtained (Table 3). An 86-fold purification of cytokinase was achieved when the specific activity of the best column fraction was compared with that of the original homogenate.

Purification of cytokinase by column chromatography with a linear gradient. Larger quantities of cytokinase were conveniently purified on a column with a linear gradient of phosphate buffer. The dialysed sample in 4 mM-phosphate buffer, pH 7.6, prepared from an extract at pH 10.5 essentially as described above, was layered on a column of DEAE-cellulose that had been equilibrated with the same buffer, and eluted with a linear gradient of 4 mM- to 0.4 M-phosphate buffer, pH 7.6. Cytokinase and protein assays were performed on those fractions that produced lysis on fibrin plates. The fractions that gave the highest specific activity for cytokinase were pooled, dialysed overnight against water at 4° and freeze-dried. A typical chromatogram is shown in Fig. 3. The first three protein peaks obtained with the stepwise elution technique were seen, with activator present in the second peak.

A maximum 50-fold purification of cytokinase was obtained by this method, with specific activities of about 3000 units/mg. of protein, assayed by the casein hydrolysis technique. With the fibrin-plate lysis assay method described by Bachmann *et al.* (1964), a 2200-fold purification of cytokinase was indicated (Ali, 1966). Freeze-drying did not affect the activator, as an 80–100% recovery of the column-fraction activity was obtained in a soluble

form by resuspending the freeze-dried material in neutral buffer. When a freeze-dried sample containing 200 units of cytokinase was assayed for activator and protease activity, against casein at pH 7.4, there was an E_{280} increment of 0.184 for activator activity and none for protease activity. Purified cytokinase did not show any proteolytic activity on fibrin plates in which the plasminogen had been inactivated by heating at 80° for 15 min.

Extinction coefficient and carbohydrate content of cytokinase. The absorption spectrum of purified cytokinase in 0.2 M-acetate buffer, pH 4, showed a distinct peak between 275 and 280 m μ ($E_{1\text{cm}}^{0.1\%} = 0.87$ at 280 m μ). A preparation of specific activity 2000 units/mg. contained 15.4 μ g. of hexose/mg. of protein and 4 μ g. of sialic acid/mg. of protein. It thus appeared that cytokinase was probably not a glycoprotein.

Gel filtration of cytokinase and determination of molecular weight. A sample of cytokinase (specific activity 1800 units/mg.) was layered on a column of Sephadex G-100 and eluted with tris-hydrochloric acid buffer, pH 7.4, containing 0.1 M-potassium chloride. The first two protein peaks eluted did not contain any activator activity. Fibrinolytic activity and cytokinase activity coincided with a third protein peak having an elution volume of 113 ml. The cytokinase specific activity increased from 1800 units/mg. to 2400 units/mg. and recovery was almost 100%.

A straight calibration line was obtained when the elution volumes of several well-characterized proteins were plotted against their molecular weights on a logarithmic scale as described by Andrews (1964) (Fig. 4). The molecular weight of

Fig. 4. Plot of elution volume against logarithm of molecular weight for proteins on Sephadex G-100. The elution volume of cytokine (○) is plotted on the calibration line obtained from an experiment with several known proteins.

urokinase was taken to be 53 000, as determined on the ultracentrifuge by Lesuk, Terminiello & Traver (1965). Cytokine, when layered on the column mixed with other proteins, still had an elution volume of 112–114 ml., and this volume almost overlapped that for human urokinase. It therefore appears that rabbit cytokine has a molecular weight in the region of 50 000.

Sedimentation behaviour of cytokine on ultra-centrifugation. A cytokine preparation (specific activity 2900 units/mg.) was centrifuged at 260 000g for 30 min., and the sedimentation pattern was analysed from a photograph. The main slow-sedimenting peak comprised 65% of the total area and had $S_{20,w}$ 2.9 s. There was a fast-sedimenting peak that comprised 12% of the total area and had $S_{20,w}$ 10.7 s. A shoulder in the main peak accounted for the remaining 23% of the total area with $S_{20,w}$ 7.5 s. When these sedimentation values were correlated with the results of the gel-filtration experiments, where cytokine had a molecular weight of about 50 000, it appeared that the slow-sedimenting peak must be that of cytokine. As there was no cytokine activity associated with any higher-molecular-weight components on gel filtration, the sedimentation peaks with higher $S_{20,w}$ values must have been due to protein contaminants.

When the sedimentation velocities for cytokine at various concentrations (3–10 mg. of protein/ml.) were plotted, a straight line was obtained. On extrapolation to zero concentration, $S_{20,w}$ 3.1 s was indicated. The extrapolated line showed slight regression with increasing protein concentration, but this was not enough to indicate whether aggregation at low concentrations of cytokine was involved.

Electrophoretic behaviour of cytokine and determination of the isoelectric region. Rabbit kidney cytokine (specific activity 2500 units/mg.) was

Fig. 5. Determination of the isoelectric region of cytokine. Samples of rabbit cytokine (○) and bovine serum albumin (●) were subjected to paper electrophoresis at various pH values, as described in the text. The migration of cytokine was determined by its fibrinolytic activity and that of albumin by staining for protein.

Fig. 6. Activation of plasminogen by cytokine, urokinase and streptokinase. Human plasminogen (50 milliunits) was incubated with different concentrations of cytokine (○), urokinase (Δ) and streptokinase (●) for 10 min., and then allowed to hydrolyse casein for 30 min. The activity is expressed as the increase in E_{280} during the casein hydrolysis, after correction for appropriate controls as described in the text.

examined at a concentration of 9mg. of protein/ml. by free moving-boundary electrophoresis. At least three migration peaks were observed. A major central peak comprised over 65% of the area, and two minor peaks on either side constituted together about 30% of the material. In the ascending limb of the apparatus, the boundary peak was descending quite rapidly towards the cathode, indicative of a protein with a high isoelectric point. Electrophoresis of cytokinase on paper was carried out at different pH values and from the migration distances (Fig. 5) an isoelectric point of pH 8.6 was obtained. That of human albumin was pH 4.8, in agreement with published values (pH 4.7-4.9).

Effect of cytokinase concentration on reaction velocity: comparison with urokinase and streptokinase. Different concentrations of cytokinase, streptokinase and human urokinase were examined for activator activity under identical conditions. It can be seen from Fig. 6 that preparations of these three enzymes could be adjusted to produce similar reaction rates and the different units of these enzymes could then be equated. On this basis 1 Christensen unit of streptokinase was approximately equivalent to 4 Ploug units of urokinase or to 40 units of cytokinase.

Heat-stability of cytokinase at different pH values. When kept at 22° for 30min. cytokinase was fairly stable over the range pH 3-11. It retained over 80% of its activity after treatment in the range pH 5-11 for 30min. at 37° and 56°, and 100% of its activity after incubation at 37° and 56° for 30min. at pH 8-9.

Effect of pH, time, temperature and cytokinase concentration on the activation of plasminogen. Cytokinase activated plasminogen maximally at pH 8.5 (Fig. 7). At concentrations of 400 units or over/2ml. assay volume, and at pH 7.4, it activated 50 milliunits of plasminogen/2ml. completely within 4min. at 37°. At or below 200 units of cytokinase/2ml., maximum activation of plasminogen required 30min. at 37°. A routine activation time of 10min. at 37° was used for the assay of cytokinase, as a longer activation time at high concentration of the enzyme could lead to auto-digestion of the plasmin formed. At 56° plasminogen was rapidly inactivated. Cytokinase was stable at this temperature at neutral pH, but was completely inactivated at 78°.

Effect of plasminogen concentration on cytokinase and urokinase activity and determination of K_m . K_m values for cytokinase and urokinase were determined graphically over a tenfold concentration range of plasminogen by the method of Lineweaver & Burk (1934). As shown in Fig. 8, straight lines were obtained when 154 units of cytokinase or about 14 Ploug units of urokinase (equivalent to 141 units of cytokinase) were used. Measurement of the

Fig. 7. Effect of pH on the activation of plasminogen by cytokinase. Cytokinase (200 units) was incubated with plasminogen (50 milliunits) in universal buffer at various pH values for 10min. After neutralization to pH 7.4, the activated plasmin was allowed to digest casein for 30min. The results are expressed as the increase in E_{280} during the casein hydrolysis.

Fig. 8. Effect of plasminogen concentration on urokinase and cytokinase activity. Rabbit cytokinase (154 units) or human urokinase (14 Ploug units) was incubated with different concentrations of human plasminogen (20-200 milliunits/2ml.) and then allowed to hydrolyse casein for 30min. The reciprocals of the velocity of hydrolysis (v = velocity, expressed in $\Delta E_{280}/\text{min.}$) and of the substrate concentration (s = concentration, expressed in milliunits of plasminogen/2ml.) were plotted to obtain a graphical estimate of the K_m values for cytokinase (●) and urokinase (▲).

slopes and intercepts indicated K_m values of 116 and 118 milliunits of plasminogen/2ml. assay volume for cytokinase and urokinase respectively.

The extrapolated straight lines for the two enzymes intercepted the abscissa at the same point and gave K_m 116 milliunits of plasminogen/2ml. (Dixon, 1953). When the concentration of plasminogen(s) was plotted against the s/v values (Lineweaver & Burk, 1934), the extrapolated straight lines for both cytokinase and urokinase intercepted the abscissa at the same point and gave K_m 117 milliunits of plasminogen/2ml. The values for V_{max} , obtained from the graphs for cytokinase and urokinase (18.9 and 16.2 units of cytokinase/min. respectively), together with the value for K_m (117 milliunits of plasminogen), were used in the Michaelis & Menten (1913) equation to give theoretical values for the velocity. These were plotted against plasminogen concentration, and it was found that the experimental points for cytokinase and urokinase fitted the theoretical curve extremely well. A perpendicular from the $V_{max}/2$ values crossed the hyperbolic curves at the same point and gave the K_m value 117 milliunits of plasminogen/2ml.

As the substrate for cytokinase is itself an enzyme, substrate concentrations are expressed in units of potential plasmin activity, rather than on a protein basis, since the specific activity can vary in different plasminogen preparations. In the experiment described the specific activity of the plasminogen preparation was 21.3 milliunits/mg. of protein. If this plasminogen preparation is assumed to be absolutely free of protein contaminants and its molecular weight is taken to be 89 000 (Robbins, Summari, Elwyn & Barlow, 1965), then the K_m for cytokinase and urokinase is $3.1 \times 10^{-5} M$.

Effect of inhibitors on the activation of plasminogen by cytokinase. Inhibition of cytokinase was elicited by adding the inhibitor to the cytokinase before its incubation with plasminogen ('inhibition of activation'); inhibition of plasmin was tested by adding the inhibitor after activation of the plasminogen with cytokinase for 10 min. but before the incubation with casein ('inhibition of plasmin').

Aminomethylcyclohexanecarboxylic acid (0.05 M) completely inhibited the activation of plasminogen by 264 units of cytokinase (Fig. 9). ϵ -Aminohexanoic acid (0.2 M) under similar conditions inhibited 80% of the cytokinase activity. Plasmin activity was inhibited 75% by 0.2 M-aminomethylcyclohexanecarboxylic acid and 40% by 0.2 M- ϵ -aminohexanoic acid.

The effect on the inhibition of cytokinase of varying the plasminogen concentrations was studied. Cytokinase (154 units) was mixed with 0.1 M- ϵ -aminohexanoic acid or 0.05 M-aminomethylcyclohexanecarboxylic acid and incubated with different concentrations of plasminogen (20–200 milliunits/2ml.). The rate of casein hydrolysis was then followed. When the reciprocals of velocity and

Fig. 9. Effect of inhibitors on cytokinase and plasmin activities. Different concentrations of ϵ -aminohexanoic acid or aminomethylcyclohexanecarboxylic acid were added to cytokinase (264 units) either before its incubation with plasminogen or after the activation of plasminogen for 10 min. Casein hydrolysis was determined as described in the text. Percentage inhibition of cytokinase by ϵ -aminohexanoic acid (●) and aminomethylcyclohexanecarboxylic acid (▲) and of plasmin by ϵ -aminohexanoic acid (○) and aminomethylcyclohexanecarboxylic acid (△) is shown.

Fig. 10. Effect of substrate concentration on the inhibition of cytokinase. Rabbit cytokinase (154 units) was either incubated directly with different concentrations of plasminogen for 10 min. (●), or first mixed with 0.1 M- ϵ -aminohexanoic acid (■) or 50 mM-aminomethylcyclohexanecarboxylic acid (▲) before the addition of plasminogen. The activated plasmin was then allowed to digest casein as described in the text. The reciprocals of the velocity (v = velocity, expressed in $\Delta E_{280}/\text{min.}$) and of the substrate concentration (s = concentration, expressed in milliunits of plasminogen/2ml.) were plotted to determine the type of inhibition.

substrate concentration were plotted (Lineweaver & Burk, 1934), the $1/v$ intercept obtained by extrapolating the values obtained in the presence of the inhibitors at high plasminogen concentrations was very similar to, but not identical with, the value in the absence of inhibitors (Fig. 10). The $-1/K_m$ value decreased, indicating possible competitive inhibition of cytokinase by ϵ -aminohexanoic acid

and aminomethylcyclohexanecarboxylic acid. The values obtained with the inhibitors at low plasminogen concentration did not fall on the straight lines, indicating that at high inhibitor/substrate ratios the inhibitor was not competitive.

Effect of thiol agents and arginine on cytokinase and urokinase activities. The effect of thiol agents on the activity of cytokinase or urokinase was determined by adding the reagent before activation of the plasminogen, and on plasmin by adding the reagent after the activation but just before incubation with casein.

Cysteine (10mM) caused about 40% inhibition of cytokinase and urokinase but had no effect on the activity of plasmin. Neither *p*-chloromercuribenzoate (0.1mM) nor iodoacetamide (5mM) had any effect on cytokinase, urokinase or plasmin activity. Higher concentrations of these substances could not be used as they absorb at 280m μ and their presence in the assay system gave high blank values. Arginine at concentrations of 50–100mM had a strong inhibitory effect on cytokinase and urokinase but little or no effect on plasmin.

DISCUSSION

With the quantitative casein-hydrolysis assay technique used in the present study, rabbit kidney was shown to be a better source of cytokinase than pig heart. The medium and conditions for extraction of the activator appeared to be different from those reported by earlier workers, who relied on the fibrin-lysis assay of cytokinase. Rabbit liver, which has been reported to be devoid of activator when fibrinolytic assay methods have been used and even to possess inhibitor activity (Albrechtsen, 1959), was found by the casein-hydrolysis technique to possess substantial cytokinase activity, especially if the lysosome-microsome fraction was separated from the cytoplasmic supernatant fraction (Ali, 1966).

The highest kidney cytokinase specific activity values were obtained from rabbits 4–5 weeks old weighing 800–1000g. Albrechtsen (1959) showed that the cytokinase content of heart, lung, kidney and spleen in the cow decreased with age, and Sandberg *et al.* (1964) showed that human infant lung was much more active than adult lung. Atencio & Reeve (1965) showed that young rabbits possessed a higher rate of catabolism and turnover of fibrinogen than did old rabbits, and it is possible that this high turnover of fibrinogen in young rabbits was related to the higher cytokinase content of their tissues.

The relationship of plasminogen activators in tissues, in blood and in other body fluids has not been determined satisfactorily. The vascular endothelium of veins and venules is now considered to be a major source of tissue activator as well as of

blood activator (Todd, 1959; Astrup & Buluk, 1963; Fearnley, 1965). Urokinase, extractable from urine, was once considered to be filtered blood activator, but has now been shown to originate in the kidney and urinary tract (Williams, 1951; Ladehoff, 1960; Painter & Charles, 1962; Buluk & Malofiejew, 1963; Ali & Lack, 1965; Holemans, McConnell & Johnstone, 1966). If the various activators were available in a purified form their biochemical and physical characterization would show whether they were similar enzymes. The purification of tissue activator from pig heart has already permitted an initial comparison to be made with purified human urokinase. The pig heart activator had a specific activity very near that of the human urokinase, and the sedimentation properties ($S_{20,w}$ 2.9–3.1s) and molecular weight were also similar (Bachmann *et al.* 1964).

Purification of the tissue activator from rabbit kidney in the present study has facilitated a more detailed comparison of cytokinase with human urokinase. Although the cytokinase preparation was heterogeneous when examined in the ultracentrifuge and by free electrophoresis, a meaningful comparison was nevertheless possible, since the major protein fraction coincided with the activator activity.

On gel filtration cytokinase behaved in a manner identical with that of human urokinase. The molecular weight of 50000 for cytokinase, calculated from the elution volume, was very near that of 54000 determined for urokinase by a similar method by Lesuk, Terminiello & Traver (1966). Urokinase analysed in the ultracentrifuge had a molecular weight of 53000 (Lesuk *et al.* 1965). White, Barlow & Mozen (1966), using sedimentation-equilibrium methods, reported a molecular weight of 54700 for a particular urokinase fraction (S2; $S_{20,w}$ 3.27s) and one of 31300 for a lower-molecular-weight fraction (S1; $S_{20,w}$ 2.66s). Burges, Brammer & Coombes (1965) reported a molecular weight for a urokinase preparation of 34500 based on gel filtration on Sephadex G-100. It appears that cytokinase is similar to the high-molecular-weight S2 fraction of urokinase obtained by White *et al.* (1966) and differs from their type S1 urokinase and other lower-molecular-weight uropepsin-degraded forms of urokinase.

The electrophoretic behaviours of cytokinase and human urokinase on starch gel at pH 6.0 were identical (Ali, 1966). On free electrophoresis at pH 4.63, cytokinase moved rapidly towards the cathode, as reported for urokinase (Ploug & Kjeldgaard, 1957; White *et al.* 1966). The isoelectric point for cytokinase (pH 8–9) may be similar to that for urokinase, which other workers have reported as a basic protein (Painter & Charles, 1962; Bergstrom, 1963).

The catalytic activation of human plasminogen by rabbit cytokinase was very similar to that by human urokinase. Cytokinase had maximum activity at pH 8.5-9.0, as had urokinase (Kjeldgaard & Ploug, 1957; Celander & Guest, 1960). The wide pH stability of cytokinase at 22° and 37° was very similar to that of urokinase. At 56°, cytokinase appeared to be more stable on the alkaline side of neutral pH, whereas urokinase has been reported to be more stable on the acid side (Kjeldgaard & Ploug, 1957); this may possibly be due to the different treatments of the enzymes during the purification procedures. The identity of the K_m values for cytokinase and urokinase, measured by several methods, is strong biochemical evidence for the similarity of these enzymes.

Aminomethylcyclohexanecarboxylic acid and ϵ -aminohexanoic acid had similar effects on cytokinase and urokinase activity (Ali, 1966). It is presumed that the type of inhibition involved is the partially competitive type as defined by Dixon & Webb (1964). ϵ -Aminohexanoic acid, besides inhibiting plasmin, trypsin and other proteases in a non-competitive manner, is also known to alter the physicochemical properties of plasminogen, plasmin fibrinogen and fibrin (Alkjaersig, Fletcher & Sherry, 1959; Iagwa, Watanabe, Amano & Okamoto, 1959; Ali, 1964; Alkjaersig, 1964; Donaldson, 1964; Maxwell & Allen, 1966). The present assay systems were not free from non-specific interference by ϵ -aminohexanoic acid and any conclusions on the type of inhibition of cytokinase by this compound are only tentative. Cysteine and arginine at various concentrations produced the same inhibitory effect on cytokinase and urokinase. The inhibition by cysteine may imply that these two enzymes require the disulphide bridge between the two half-cystine residues to be intact for their maximum activity.

It appears from our experimental results and from comparison with other published work that rabbit kidney cytokinase and human urokinase are similar, if not identical, enzymes. The kidney therefore appears to play an important role in the synthesis or storage or both of tissue activator (cytokinase) and urokinase. It should now be possible to use the extraction and purification procedure described in this study to prepare the activator from other tissues. If blood activator can be obtained in purified and stable form it should be possible to establish the interrelation of various physiological activators and their site of origin.

We are indebted to Dr K. W. Walton and Dr D. Norman-Sell of the Department of Experimental Pathology, University of Birmingham, for facilitating the ultracentrifugal and free-electrophoresis studies. We thank the American Red Cross Society for Cohn fraction III, Leo Pharmaceutical

Products for an ampoule of urokinase, and A. B. Kabi for the inhibitors and other blood products.

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