up-to-date information and critical reviews, abstracts of important papers recently published by sister journals, and more streamlined less discursive articles, we could take a bold step forward in reducing the volume of reading material for our long-suffering colleagues. We would hope also to co-operate with other editors in defining the field of interest in our journals and at all times attempt to cater at least as much for the reader as for the author.

We may feel that these problems are becoming more and more urgent in the medical sciences, but more than two thousand years ago, in the heyday of Pompeii, the matter was already causing concern. On a wall of the Meeting House which was used as a wall newspaper there is a clear inscription, which freely translated reads: "It is a miracle O wall that thou has not yet crumbled under the weight of so much written nonsense."

REFERENCES

- Abercrombie, M. L. Johnson (1960). *Anatomy of Judgment*, p. 57. Hutchinson, London.
 Bacharach, A. L. (1962). *Nature (Lond.)*, **195**, 236.
 Bernal, J. D. (1939). *The Social Function of Science*. London.
 Lewis, Sir Thomas (1944). *Lancet*, **1**, 619, 649, 685, 696.
 Rutstein, D. D. (1961). *New Engl. J. Med.*, **265**, 321.
 Shiloh, Ailon (1962). *New Scientist*, **284**, 169.
 Thorpe, W. V. (1962). *Nature (Lond.)*, **195**, 236.
 Walshe, Sir Francis (1959). *Perspectives in Biology and Medicine*, **2**, 197.

IN-VITRO AND IN-VIVO STUDIES OF A PREPARATION OF UROKINASE

BY

G. P. McNICOL, M.B., M.R.C.P.G.
M.R.C.P.Ed.

Honorary Senior Registrar

SANDRA B. GALE, B.Sc.
Scientific Assistant

AND

A. S. DOUGLAS, M.D., B.Sc., F.R.C.P.Ed.
M.R.C.P., M.R.C.P.G.

Senior Lecturer in Medicine and Honorary Consultant
Physician

From the University Department of Medicine,
Royal Infirmary, Glasgow

At the present time there is much interest in the possible use of fibrinolytic enzymes in the treatment of intra-vascular thrombosis. One such enzyme, urokinase, is the physiological fibrinolytic substance present in a normal urine. Urokinase owes its fibrinolytic activity to its property as an activator of plasminogen (Fig. 1). Plasminogen, a natural plasma globulin, inactive in its native state, is converted by urokinase to plasmin, a proteolytic enzyme with wide substrate specificity. As suggested by Sherry *et al.* (1959a), clot lysis *in vivo* probably takes place by diffusion of activator—for example, plasma activator—into the thrombus, with conversion of clot plasminogen to plasmin. The plasmin so produced is close to the substrate of choice, fibrin, which is digested. Inhibitory mechanisms in the plasma normally prevent the appearance of free plasmin in the circulation.

Urokinase, itself a proteolytic enzyme, activates plasminogen by first-order kinetics probably by splitting lysine and/or arginine bonds (Kjeldgaard and Ploug, 1957; Alkjaersig *et al.*, 1958). It is a colourless protein with a high degree of stability over a wide pH range (Ploug and Kjeldgaard, 1957).

Bjerrehuus (1952) has shown that urokinase concentrations are similar in bladder urine and urine obtained from the renal pelvis. The work of von Kaulla and Swan (1958) and of Smyrniotis *et al.* (1959) suggests that urokinase may represent in part at least excreted plasma plasminogen activator. There is evidence to support the concept that it has a physiological role in maintaining the patency of the urinary tract by promoting the lysis of fibrinous deposits (McNicol *et al.*, 1961).

Because of its human origin, it has been hoped that urokinase might have many advantages, including freedom from toxicity and antigenicity, as a therapeutic fibrinolytic substance (*British Medical Journal*, 1962). Interest in the use of urokinase was stimulated by its isolation and purification by Ploug and Kjeldgaard (1957). There are reports in the literature of the use of urokinase-activated plasmin—for example, Sokal *et al.* (1958) and Sussman and Fitch (1958)—and Hansen *et al.* (1961) also report on the infusion of urokinase to 22 subjects, though they present no data on the biochemical effects induced.

Recently a commercial preparation of urokinase, intended for trial by clinical investigators, has become available (Leo urokinase), and the present paper gives the results of study of this preparation *in vitro* and *in vivo*.

Materials and Methods

Urokinase.—A purified preparation, isolated from the urine of normal males, was made available by Leo Laboratories Ltd. The preparation, supplied as sterile yellowish-white pellets, was freely soluble in 0.9% sodium chloride. The manufacturers state that potency, in arbitrary units (Ploug and Kjeldgaard, 1957), varies from batch to batch from 5,000 to 12,000 units/mg. The materials used in this study had a potency of 7,100 units/mg. Calcium content of a 10,000-unit/ml. solution was less than 0.2 mEq/litre.

Fibrinogen was prepared from the expired bank plasma by a phosphate buffer method (Biggs and Macfarlane, 1962a).

¹³¹I-tagged fibrinogen was prepared by the method of Clement and McNicol (1959). Clottable radioactivity varied from 90 to 93%. Samples were counted in a well-type scintillation counter with a pulse height analyser.

Plasminogen.—A preparation of human plasminogen, soluble at neutral pH, was made available by the Kabi Pharmaceutical Company.

Thrombin.—Parke Davis thrombin topical.

Citrated Blood.—This was collected by clean venepuncture. Nine volumes of blood were mixed with one volume of 3.8% sodium citrate in a graduated centrifuge tube.

Plasminogen assays were carried out by the caseinolytic technique of Remmert and Cohen (1949) as modified by Alkjaersig *et al.* (1959a).

Activator assay, with plasminogen-enriched ¹³¹I-tagged clots, was performed by the technique of

FIG. 1.—Showing how urokinase converts plasminogen to plasmin. Plasmin, a proteolytic enzyme with wide substrate specificity, digests fibrin to give soluble products.

Aljaersig *et al.* (1959b), as modified by McNicol *et al.* (1962), with the further modification that the radioactive clots were incubated with the test plasma for one hour instead of 30 minutes. In this assay system the test plasma is incubated with plasminogen-enriched ^{131}I -labelled plasma clots; radioactivity released from the clots during incubation is a measure of the activator content of the test plasma.

Whole-clot lysis times were determined by clotting 0.5 ml. of whole blood with 0.1 ml. of thrombin, 10 N.I.H. units/ml., incubating at 37° C., and measuring time for complete clot lysis.

Euglobulin Lysis Times.—The method of Nilsson and Olow (1962) was used. In fibrinolytic assays it has been shown (Sherry and Alkjaersig, 1957) that activity is a direct function of the reciprocal of the lysis time, and accordingly a logarithmic plot of lysis time against units of activity shows a linear relationship.

$$(\text{Activity} = \frac{\text{constant}}{\text{time}})$$

$$\therefore \text{Activity} \times \text{time} = \text{constant}$$

$$\therefore \log (\text{activity} \times \text{time}) = \text{constant}$$

$$\therefore \log \text{activity} + \log \text{time} = \text{constant}$$

As suggested by Sherry *et al.* (1959b), euglobulin lysis times can usefully be expressed in terms of arbitrary units of activity derived from such a plot. In the present study a lysis time of 300 minutes has been assigned an activity of one unit. The values for other lysis times are obtained by dividing the lysis time observed into 300.

One-stage "prothrombin" times were carried out as described by Douglas (1962).

Fibrinogen assays were carried out by the method of Ratnoff and Menzie (1951) as modified by Alkjaersig (1960).

Thrombin clotting-times were performed by the method of Fletcher *et al.* (1959).

Recalcification times were estimated as described by Biggs and Macfarlane (1962b). In some of the experiments the plasma was low-spun, and in others, where it was high-spun, cephalin was added to the system in place of saline. In all cases epsilon aminocaproic acid (E.A.C.A.) (see below) was present in a concentration of 10^{-2} M to inhibit lysis.

Antihaemophilic Globulin and Christmas Factor Assays.—The method, to be described elsewhere, is dependent on the clotting-time of recalcified plasma from patients with severe haemophilia (or Christmas disease) which is maximally "contact activated" by kaolin. Cephalin, prepared as described by Bell and Alton (1954), is added to the substrate system.

Factor V assay was performed as described by Douglas and Biggs (1953) except that the readings were taken only at the five- and six-minute points after the start of incubation. Results are expressed as a percentage of the preinfusion value.

Fibrin plates were made up as described by Nilsson and Olow (1962) using highly purified plasminogen-rich bovine fibrinogen supplied by Dr. I. N. Gröndahl of Stockholm. Twenty-five ml. of 0.1% fibrinogen solution in "tris" buffer, 0.15 M, pH 7.8, is clotted with 1 ml. of thrombin, 20 N.I.H. units/ml., in a "perspex" plate, internal diameter 11.5 cm. Then 30- μl . aliquots of test solution are pipetted on to the surface of the plate, which is incubated for 18 to 20 hours at 37° C. Lytic activity is recorded in square millimetres, as the

product of two perpendicular diameters of the area of lysis.

Electrophoresis.—Oxoid cellulose acetate strips were used for electrophoresis. Strips were run for two hours at 150 volts in a Shandon horizontal electrophoresis tank. After staining, strips were scanned in a Chromoscan recording and integrating densitometer.

Esterolytic assays, using tosyl arginine methyl ester (TAME), were carried out by the method of Troll *et al.* (1954). Where serum was used in the assays it was dialysed with constant stirring overnight at 4° C. against a large volume of 0.9% saline. Optimum urokinase concentration was found to be 200 units/ml., and incubation was for 30 minutes.

Urokinase-sensitivity Tests.—To 0.2 ml. of citrated plasma is added 80 μl . of urokinase solution, 500 units/ml., and thrombin, 0.1 ml. of a 20 N.I.H. unit/ml. solution. The plasma is incubated at 37° C. and the times for complete clot lysis are measured.

Thromboplastin generation tests were carried out as described by Biggs and Douglas (1953), with the following modifications. The volumes of plasma, serum, platelet suspension, M/40 calcium chloride and urokinase (10,000 units/ml. solution) were 0.3 ml. In each case saline was added to give a constant volume of 1.5 ml. and E.A.C.A. was present in a concentration of 10^{-2} M to inhibit lysis.

Epsilon aminocaproic acid (E.A.C.A.) was supplied by Kabi Pharmaceuticals and by Boots. At a concentration of 10^{-2} M, E.A.C.A. inhibits both plasminogen activation and plasmin (Alkjaersig *et al.*, 1959a).

Results of In-vitro Studies

Fibrinolytic Activity

In purified systems the urokinase preparation was found to be a potent activator of plasminogen. For example, in the fibrin plate test urokinase in a concentration of 10 units/ml. gave a zone of lysis of 404 ± 49 sq. mm. (mean and standard deviation of 24 observations). No lysis was seen when the plates contained the fibrinolytic inhibitor E.A.C.A. at a concentration of 5×10^{-3} M, a level adequate to inhibit plasminogen activation but not to inhibit proteolytic activity (Alkjaersig *et al.*, 1959a). In the radioactive clot assay potent activity was also seen at a urokinase concentration of 5 units/ml.

As has been reported by Sherry *et al.* (1959a), urokinase possesses esterolytic activity against TAME; the present preparation demonstrated this, 1 mg. of the urokinase preparation producing lysis of 161 ± 23 μM TAME in 30 minutes (mean and standard deviation of eight observations).

Inhibition of Urokinase

It was observed that in systems containing serum or plasma, also *in vivo* (see below), the activity of urokinase was much less than that seen with similar amounts of urokinase in purified systems. Fig. 2 shows the effects of plasma in inhibiting urokinase in the fibrin plate test. Mean values and standard deviations from observations on 18 plasmas are shown. Complete inhibition of one unit of urokinase was observed when 50 μl . or more of plasma was added to the urokinase preparation before assay. Similar results were found in the radioactive clot system, where in each of 20 experiments inhibition was complete with 50 μl . or more of plasma per unit of urokinase; with 10 μl . of plasma per unit of urokinase inhibition was $22 \pm 10\%$.

To identify the protein fraction responsible for this inhibitory effect, electrophoresis of 10 μ l. of serum was carried out on cellulose acetate in barbitone buffer, 0.1 M, pH 8.6. After electrophoresis the wet cellulose acetate paper was divided longitudinally—that is, at right angles to the origin. One half of the paper was stained with Ponceau S; the other half was cut, parallel

FIG. 2.—Effect of plasma in inhibiting urokinase in the fibrin plate test. Mean values and standard deviation from observations on 18 plasmas are shown.

FIG. 3.—Effect of various serum fractions, obtained by electrophoresis on cellulose acetate, in inhibiting urokinase in the fibrin plate test.

serum on urokinase was destroyed by lowering pH (pH 2 for 15 minutes). It was partially but not completely precipitated by 33 $\frac{1}{3}$ and 50% saturation with ammonium sulphate. It was not adsorbed on kaolin.

As the action of urokinase in the fibrin plate and on the radioactive clot is mediated through its ability to convert plasminogen to plasmin, and it is the plasmin so produced which brings about the lysis observed in the assay systems, the inhibitory effects seen may be due in part at least to the antiplasmin content of the plasma or serum. In order to demonstrate an "anti-urokinase" as opposed to an antiplasmin effect in serum, the following experiment was carried out using a caseinolytic assay. Urokinase (0.25 ml. of a 25 unit/ml. solution) was incubated at 37° C. for one hour with 0.5 ml. of plasminogen solution, 7 casein units/ml. After addition of 0.5 ml. of serum the caseinolytic activity induced was measured. The assay was also

carried out adding the serum first, and incubating the serum at 37° C. for one hour with the urokinase before adding the plasminogen solution. Twenty-five sera were examined in this way. Mean caseinolytic activity for the 25 experiments (when plasminogen was added first) was 0.12 ± 0.14 casein unit greater than when serum was added first.

Serum was also found to inhibit the esterolytic activity of urokinase on TAME. Mean esterolytic activity of 1 mg. of urokinase in eight experiments was 161 ± 23 μ M TAME lysed in 30 minutes. When serum, 20 μ l./unit of urokinase, was added, mean esterolytic activity per mg. urokinase was in eight experiments 111 ± 57 μ M TAME. The difference is significant ($t=2.2$, $P<0.05$).

Urokinase-sensitivity Tests.—In 16 subjects mean lysis time was 13.2 ± 9.1 minutes.

Thromboplastic or Coagulative Activity

Since human urine is known to contain thromboplastic activity, it was decided to study the coagulative property of the urokinase preparation. Urokinase in increasing concentrations was substituted for saline in a recalcification time assay, using fresh normal plasma as substrate and incorporating E.A.C.A. at a concentration of 10^{-2} M to inhibit lysis. A progressive reduction in the recalcification time, linearly related to urokinase concentration in a semilogarithmic plot, was found, and is shown in Fig. 4. Substituted for brain extract in the one-stage prothrombin time, it was found that the urokinase preparation in a concentration of 10,000 units/ml. had a similar potency to a 1/750 dilution of the brain extract at present in use in this laboratory.

A shortening of the recalcification time by the urokinase in the presence of E.A.C.A. was repeatedly demonstrated in fresh normal plasma. This coagulative

FIG. 4.—Reduction in clotting-time with increasing amounts of urokinase added to normal plasma before recalcification.

TABLE I.—Reduction in Recalcification Time of Hageman Plasma by Urokinase. Tests were Carried Out in the Presence of E.A.C.A. at a Concentration of 10^{-2} M to Inhibit Lysis

Approximate Concentration of Urokinase (Units/ml.) in Test System	Recalcification Time (Seconds)
1,500	130
750	138
375	150
185	220
90	250
45	315
20	335
10	461
5	540
2	585
Saline	825

TABLE II.—Effect of Urokinase (10,000 Units/ml.) in the Thromboplastin Generation Test in Partially Correcting Defects Due to Antihaemophilic Globulin, Christmas Factor, and Platelet Deficiency

Incubation time, minutes:	Thromboplastin Generation Test Clotting-times (Seconds)					
	1	2	3	4	5	6
Normal plasma. Normal serum. Platelets	30	10.5	10.5	11	11	11
Normal plasma. Christmas serum. Platelets	171	141	139	140	117	84
	115	100	96	80	70	54
Haemophilic plasma. Normal serum. Platelets	35	29	31	31	30.5	31
	74	42	45	26	26.5	26
Normal plasma and serum (diluted 1:4). Platelets	113	80	46	27	21	17
Normal plasma and serum (diluted 1:4). No platelets	158	149	141	140	127	101
	112	106	100	92	92	88

UK = Urokinase.

effect became less obvious when the test plasma was kept in glass tubes at room temperature, as the saline control clotting-time shortened owing to the contact effect. The coagulative property of the urokinase was readily demonstrated in plasma collected and clotted in silicone tubes. In such an experiment

the addition of urokinase at a concentration of 1,500 units/ml. reduced the recalcification time from 450 to 175 seconds.

In an experiment using Hageman plasma the reductions in the recalcification times were very readily observed when small concentrations of the urokinase preparation were present in the system. The figures obtained are shown in Table I.

Table II shows the effect of uro-

Fig. 5.—Separation of urokinase preparation by electrophoresis into at least four components in the buffer system used (acetate buffer, 0.1 M, pH 4.5). These components have differing levels of fibrinolytic activity and may have differing coagulative properties.

kinase in the thromboplastin generation test in partially correcting for antihaemophilic globulin deficiency, Christmas factor deficiency, and platelet deficiency. Urokinase was found to substitute partially for Christmas factor and to a slight extent for antihaemophilic globulin. In the test for substitution of urokinase for platelets no correction was seen when the plasma or serum was tested at the standard dilution, but when plasma and serum were further diluted 1:4 slight correction of the abnormality resulting from platelet lack was seen.

Electrophoretic Studies

To attempt separation of fibrinolytic from coagulative activity the urokinase preparation was studied after electrophoresis. Acetate buffer, pH 4.5, 0.1 M, was found to give the best separation. After electrophoresis of 20 μ l. of urokinase solution, 10,000 units/ml., on cellulose acetate, each paper was divided, at right angles to the origin, into three equal portions. One portion

was stained with nigrosine, the other two were cut parallel with the origin into strips 1 cm. wide. Each strip from the second portion of the paper was applied to the surface of a fibrin plate and the extent of lysis after overnight incubation was assessed. To each strip from the third portion of the paper was added, in a 12 by 75 mm. test-tube, 0.2 ml. of saline. Each strip was pounded with a glass rod for several minutes; the strips did not disintegrate. Thirty minutes later the ability of 0.1 ml. of the eluting fluid to shorten the recalcification time of normal plasma was measured. Results are shown in Fig. 5. In this buffer system urokinase separates into at least four components. The fibrinolytic activity is largely associated with slow-moving components, whereas the coagulation property is more uniformly distributed.

Results of In-vivo Studies

The urokinase preparation was infused intravenously, diluted with from 250 to 500 ml. of saline, in five adult subjects (three women and two men). Details are set out in Table III. All subjects were given 100 mg. of hydrocortisone hemisuccinate with the urokinase infusion.

TABLE III.—Details of Urokinase Dosage

Subject No.	Dose of Urokinase	Total
1	15,000 units hr. for 2 hours	30,000
2	40,000 " " 1 hour	40,000
3	40,000 " " 2 hours	80,000
4	80,000 " " 1 hour	80,000
5	60,000 units over 30 minutes	60,000

Subject 1, given 30,000 units over two hours, showed no detectable changes in fibrinolytic activity or coagulation assays (fibrin plate test, radioactive clot assay, euglobulin lysis time, plasminogen, fibrinogen, prothrombin time, thrombin time).

The effects of infusion of 40,000 units of urokinase over one hour in Subject 2 are shown in Fig. 6. At the end of the infusion the radioactive clot test, the fibrin plate test, and the euglobulin lysis time all showed evidence of activator in the circulation. There was a slight fall in plasminogen, but no significant changes occurred in prothrombin time, thrombin time, fibrinogen,

Fig. 6.—Effect in Subject 2 of infusion of 40,000 units of urokinase over one hour (shown with hatching).

recalcification time, antihæmophilic globulin (A.H.G.), and Christmas factor assays. Whole-clot lysis times were never less than 24 hours.

Fig. 7 shows the effect of infusion of 40,000 units of urokinase/hour for two hours in Subject 3. At the end of one hour activator activity was seen in the radioactive clot assay and euglobulin lysis was accelerated; no activity was seen in the fibrin plate test. At two hours there was a 50% fall in plasminogen, and the radioactive clot assay showed increased activity; lytic activity was also seen in the fibrin plate test. There were no significant changes in thrombin time, prothrombin time, and fibrinogen levels. Whole-clot lysis times were never less than 24 hours. In an A.H.G. assay clotting-times fell from 171 seconds before infusion to 160 seconds at the end of infusion. Clotting-times in the Christmas factor assay fell from 149 seconds before infusion to 105 seconds at the end of infusion.

In Subject 4 infusion of 80,000 units in one hour produced activity in the fibrin plate test and acceleration

FIG. 7.—Effect in Subject 3 of infusion of 80,000 units of urokinase over two hours (shown with hatching). Attention is particularly drawn to the reduction in recalcification time.

FIG. 8.—Effect in Subject 4 of infusion of 80,000 units of urokinase over one hour (shown with hatching). Reduction in recalcification time is also seen in this subject. Results of other coagulation assays are set out in Table IV.

D

of the euglobulin lysis time (Fig. 8). There were no significant changes in thrombin time, prothrombin time, plasminogen, or fibrinogen. Whole-clot lysis times were never less than 24 hours. Recalcification time (cephalin added) fell from 82 to 37 seconds. The changes in A.H.G. assay, Christmas factor assay, and factor V assay are set out in Table IV. In A.H.G. and Christmas assays, apparent A.H.G. and Christmas activities were increased—that is, clotting times were shortened. There was also a sharp fall in factor V activity.

TABLE IV.—Antihæmophilic Globulin, Christmas Factor, and Factor V Assays in Subject 4 (See Text). Urokinase (80,000 Units) was Given Intravenously for the First Hour

	Time (Hours)					
	0	1	2	3	4	21
A.H.G. assay (seconds)	126	85	86	67	119	111
Christmas factor assay (seconds)	115	66	56	62	106	110
Factor V (%)	100	55	45	18	100	100

Subject 5 had an infusion of 60,000 units over 30 minutes (Fig. 9). The radioactive clot assay, the fibrin plate test, and the euglobulin lysis time all indicated a marked increase in activator activity. Peak activity in the radioactive clot assay was 970 counts/100 seconds. A urokinase solution 20 units/ml. gave 6,800 counts/100 seconds; 20 units/ml. is approximately the concentration which should have been induced in the plasma by rapid infusion of 60,000 units. Whole-clot lysis times were never less than 24 hours.

FIG. 9.—Effect in Subject 5 of infusion of 60,000 units of urokinase over 30 minutes (shown with hatching). In this subject no coagulative activity was seen.

Effects on Euglobulin Lysis Times and Whole-clot Lysis Times.—The maximum increase in euglobulin lysis seen in each of these five subjects was 0, 0.6, 0.8, 1.6, and 1.7 units respectively. The mean of these values is 0.94 unit. Six healthy young male colleagues were exercised very vigorously for five minutes (skipping with a heavy rubber tube). The mean increase in euglobulin lysis found after exercise in these subjects was 3.5 units (range 1.2 to 6.0 units). None of the five subjects given urokinase showed at any time whole-clot lysis times of less than 24 hours. The whole-clot lysis times found in samples taken from the six subjects after exercise were respectively 385 minutes; between 9 and 21 hours; 147

minutes; more than 24 hours; between 9 and 24 hours; and 213 minutes.

Urokinase-sensitivity Tests.—For these five subjects lysis times in the urokinase sensitivity test were before infusion 4 min. 55 sec., 12 min. 15 sec., 14 min. 25 sec., 10 min. 30 sec., and 9 min. 40 sec. respectively, and were 5 min. 25 sec., 10 min. 45 sec., 12 min. 10 sec., 11 min. 30 sec., and 8 min. 55 sec. in samples taken 10 days after the urokinase infusion.

Urinary Urokinase Activity.—In Subjects 3 and 5 urinary urokinase activity was measured by the fibrin plate test for a period of three hours during and after urokinase infusion. Urinary urokinase activity was also measured for a three-hour period at the same time on a control day. Activity was respectively 26 and 35% higher in the two subjects on the day of urokinase infusion than on the control day.

Toxicity.—No side-effects were noted with the urokinase infusions. No symptoms were produced and there were no alterations in temperature or pulse rate.

Antigenicity.—Subject 1 was given intravenously a challenging dose of 1 mg. of urokinase one week after the initial infusion of 30,000 units. One further week later intradermal injection of 10 μ g. produced no skin reaction. Serum obtained at the time of the intradermal test was examined by Dr. Robert Goudie for precipitating antibody, using double diffusion in an agar-gel plate. The serum was used undiluted, and the antigen, urokinase, was tested at 1 and 0.1 mg./ml. No precipitins were found after seven days' diffusion at 20° C. It should also be noted that no significant changes in urokinase sensitivity tests were found in the five patients in samples taken off 10 days after the urokinase infusion.

Discussion

In-vitro testing of the urokinase preparation demonstrates that it is an effective activator of the plasminogen-plasmin system. In the fibrin plate test no activity was seen in the presence of E.A.C.A. at a concentration of 5×10^{-3} M, which inhibits plasminogen activation. Esterolytic activity against a synthetic substrate was shown by the present preparation, 1 mg. of which lysed 161 ± 22 μ M TAME in 30 minutes.

The inhibitory action of plasma and serum on urokinase, previously reported by Hansen *et al.* (1961) and Paraskevas *et al.* (1962), can readily be demonstrated in the fibrin plate test and the radioactive clot test.

As all activator assays measure the plasmin produced by plasminogen activation, inhibition can be due either to antiplasmin activity or to antiactivator activity; and the inhibitory effect seen in the present studies appears to be largely localized in the α_1 - and α_2 -globulin fractions to which Norman and Hill (1958) have demonstrated that the antiplasmins belong. The caseinolytic assays in which, with sera from some subjects, less casein is digested when urokinase and serum are incubated together before addition of further purified plasminogen than when serum is added after plasminogen, suggest that the inhibitory effect is due, in part at least, to direct inhibition of plasminogen activation. The finding in the esterolytic assays that some sera inhibit the action of urokinase on TAME reinforces the view that some sera contain antiactivator activity. However, as sera also contain plasminogen and antiplasmin, the results must be regarded only as strongly suggestive of the existence of antiurokinase activity in plasma and serum.

Thromboplastic or Coagulative Activity

As shown in Fig. 5 the preparation is not a homogeneous protein; there can be no doubt from the experiments that the preparation can be separated into components with much lytic activity and others with less or none. There is suggestive but not final evidence that the coagulative activity may be separated from the lytic activity.

Whole normal urine is known to contain thromboplastic activity (Tocantins and Lindquist, 1947). Comparison of the lytic and thromboplastic activities of normal urine would indicate that much thromboplastic activity has been removed from this urokinase preparation, but it still possesses some coagulative property. Though the thromboplastic activity seen *in vitro* in the recalcification time and the thromboplastin generation test with a solution of 10,000 units/ml. is not potent (equivalent to a 1:750 dilution of brain extract in the prothrombin time), definite evidence of coagulative activity was seen *in vivo* in Subjects 3 and 4, where plasma concentration of urokinase can have been at most 25 units/ml. In these subjects infusion of urokinase was followed by shortening of recalcification time and an apparent rise in A.H.G. and Christmas factor levels, probably by distortion of the assay system by the non-specific coagulative activity of the urokinase. The fall in factor V activity in Subject 4 may perhaps be most readily explained on the basis of factor V consumption due to some measure of intravascular coagulation.

Iatridis *et al.* (1960) found that a urokinase preparation had a "Hageman-like" effect. In terms of current knowledge there would seem to be two possible explanations of the coagulative phenomena observed in our experiments—either inadequate removal of the thromboplastin component of urine or a Hageman-like effect of the urokinase molecule itself. Since there can be no doubt that the current preparation is not a homogeneous protein, it might well be possible to separate the preparation into its several components on a larger scale; the fractions obtained could then be tested for coagulative activity and lytic activity to determine whether a product can be obtained which has lytic activity but no coagulative properties.

It is perhaps surprising that with the dosage used in our experiments *in vivo* it was possible to demonstrate a coagulative effect. The generation of thrombin from prothrombin is autocatalytic, and therefore prothrombin conversion, once triggered by the effect demonstrated *in vitro*, may have been perpetuated by the autocatalytic reaction *in vivo*.

Fibrinolytic Effects *In Vivo*

In urokinase sensitivity tests it was found that, *in vitro*, a concentration of 200 units/ml. of plasma gave a mean lysis time of 13 minutes. In fibrinolytic therapy with streptokinase it is usually thought desirable to induce in the plasma a brisk fibrinolytic state, and initial doses of streptokinase are calculated to produce plasma clot-lysis times of 10 to 30 minutes (Sherry *et al.*, 1959b; Nilsson and Olow, 1962; McNicol *et al.*, 1962). On the basis of the urokinase sensitivity tests, and assuming a plasma volume of 3 litres, a dose of about 600,000 units would be necessary to produce such activity. Hansen *et al.* (1961) found that at least 250,000 units of urokinase had to be administered to produce whole-plasma-clot lysis times of less than three hours. It is

not therefore surprising that in the present study only slight or moderate fibrinolytic activity, less than one-third of that induced in healthy subjects by strenuous exercise, was seen after infusion of 30,000–80,000 units of urokinase. In view of the coagulative activity seen *in vivo* in Subjects 3 and 4, there must be some doubt about the wisdom of administering doses adequate to produce brisk fibrinolytic activity.

It may be, however, that with larger doses of urokinase any fibrin deposited will be so rapidly lysed that no untoward clinical effects will be produced.

Summary

A commercial preparation of human urokinase, the physiological fibrinolytic substance present in normal urine, has recently become available for clinical trial as a fibrinolytic agent in the treatment of intravascular thrombosis. The present paper gives an account of *in-vitro* and *in-vivo* study of this preparation (Leo urokinase). *In vitro* in purified systems the preparation is a potent plasminogen activator, but, because of an inhibitor in plasma, relatively large doses are required to produce fibrinolytic activity *in vivo*. As normal urine has thromboplastic properties the preparation was also examined for coagulative activity, and was found to shorten the recalcification time of fresh normal plasma and of Hageman plasma. On electrophoresis the preparation has at least four components; the fibrinolytic activity is largely located in one specific band. The preparation was infused intravenously in five subjects in an amount which produced only slight fibrinolytic activity in the plasma; in two subjects biochemical but not clinical evidence of coagulative activity *in vivo* was observed. There was no evidence of antigenicity. In view of the coagulative property of the preparation it would seem possible that doses adequate to produce brisk fibrinolytic activity in the circulation may be associated with some hazard; with further purification and elimination of the coagulative component the preparation would be an important agent for clinical trial.

Our thanks are due to Professor E. M. McGirr for his interest in this work and his advice in the preparation of this paper. We are indebted to the Medical Research Council for financial support. Dr. Robert Goudie kindly carried out the precipitin tests. Thanks are also due to Dr. Austin Darragh for supplies of urokinase, to Dr. Hans Dahlström for plasminogen and E.A.C.A., to Dr. E. V. B. Morton for E.A.C.A., to Miss B. C. Bayley for technical assistance, to Mr. R. MacGregor for photography, and to Dr. W. J. Jenkins for Hageman plasma.

REFERENCES

- Alkjaersig, N. (1960). In *N.I.H. Conference on Thrombolytic Agents*, edited by H. R. Roberts and D. Geraty, p. 316. Chapel Hill.
- Fletcher, A. P., and Sherry, S. (1958). *J. biol. Chem.*, **233**, 86.
- — — (1959a). *Ibid.*, **234**, 832.
- — — (1959b). *J. clin. Invest.*, **38**, 1086.
- Bell, W. N., and Alton, H. G. (1954). *Nature (Lond.)*, **174**, 880.
- Biggs, R., and Douglas, A. S. (1953). *J. clin. Path.*, **6**, 23.
- and Macfarlane, R. G. (1962a). *Human Blood Coagulation and its Disorders*, 3rd ed., p. 372. Blackwell, Oxford.
- (1962b). *Ibid.*, p. 384.
- Bjerrehuus, I. (1952). *Scand. J. clin. Lab. Invest.*, **4**, 179.
- Brit. med. J.*, 1962, **1**, 1398.
- Clement, W. E., and McNicol, G. P. (1959). *J. clin. Path.*, **12**, 544.
- Douglas, A. S. (1962). *Anticoagulant Therapy*, p. 279. Oxford.
- and Biggs, R. (1953). *Glasg. med. J.*, **34**, 329.
- Fletcher, A. P., Alkjaersig, N., and Sherry, S. (1959). *J. clin. Invest.*, **38**, 1096.
- Hansen, P. F., Jorgensen, M., Kjeldgaard, N. O., and Ploug, J. (1961). *Angiology*, **12**, 367.
- Iatridis, S. G., Wilson, E. G., Ferguson, J. H., and Rierison, H. A. (1960). *Thrombos. Diathes. haemorrh. (Stuttg.)*, **5**, 50.
- von Kaulla, K. N., and Swan, H. (1958). *J. thorac. Surg.*, **36**, 519.
- Kjeldgaard, N. O., and Ploug, J. (1957). *Biochim. biophys. Acta (Amst.)*, **24**, 283.
- McNicol, G. P., Douglas, A. S., and Bayley, C. (1962). *Lancet*, **2**, 1297.
- Fletcher, A. P., Alkjaersig, N., and Sherry, S. (1961). *J. Lab. clin. Med.*, **58**, 34.
- Nilsson, I. M., and Olow, B. (1962). *Acta chir. scand.*, **123**, 247.
- Norman, P. S., and Hill, B. M. (1958). *J. exp. Med.*, **108**, 639.
- Paraskevas, M., Nilsson, I. M., and Martinsson, G. (1962). *Scand. J. clin. Lab. Invest.*, **14**, 138.
- Ploug, J., and Kjeldgaard, N. O. (1957). *Biochim. biophys. Acta (Amst.)*, **24**, 278.
- Ratnoff, O. D., and Menzie, C. (1951). *J. Lab. clin. Med.*, **37**, 316.
- Remmert, L. F., and Cohen, P. P. (1949). *J. biol. Chem.*, **181**, 431.
- Sherry, S., and Alkjaersig, N. (1957). *Thrombos. Diathes. haemorrh. (Stuttg.)*, **1**, 264.
- Fletcher, A. P., and Alkjaersig, N. (1959a). *Physiol. Rev.*, **39**, 343.
- Lindemeyer, R. I., Fletcher, A. P., and Alkjaersig, N. (1959b). *J. clin. Invest.*, **38**, 810.
- Sokal, J. E., Ambrus, J. L., and Ambrus, C. M. (1958). *J. Amer. med. Ass.*, **168**, 1314.
- Smyrniotis, F. E., Fletcher, A. P., Alkjaersig, N., and Sherry, S. (1959). *Thrombos. Diathes. haemorrh. (Stuttg.)*, **3**, 257.
- Sussman, B. J., and Fitch, T. S. P. (1958). *J. Amer. med. Ass.*, **167**, 1705.
- Tocantins, L. M., and Lindquist, J. N. (1947). *Proc. Soc. exp. Biol. (N.Y.)*, **65**, 44.
- Troll, W., Sherry, S., and Wachman, J. (1954). *J. biol. Chem.*, **208**, 85.

FIBRINOLYTIC THERAPY OF CORONARY THROMBOSIS CONTROLLED TRIAL OF 75 CASES

BY

H. A. DEWAR, M.D., F.R.C.P.
P. STEPHENSON, M.B., M.R.C.P.
A. R. HORLER, M.D., M.R.C.P.
A. J. CASSELLS-SMITH, M.D.

AND

P. A. ELLIS, M.B., M.R.C.P.Ed.
The Royal Victoria Infirmary, Newcastle upon Tyne

In a pilot study Dewar *et al.* (1961) described the experimental work upon which the use of fibrinolysis in the treatment of coronary thrombosis is based, reviewed the experiences of other workers in this field, and described the methods employed in the present trial. At that time 33 patients, 17 of them treated and 16 controls, had been included and the preliminary results did not disclose any striking difference between the two groups except perhaps in some features of the serum transaminase curves which succeeded the infarction. The preparation used was "thrombolysin," consisting of human plasminogen (fibrinolysin), activated by streptokinase and claimed to be devoid of "free activator." This has been shown to be active in lysis *in vitro*, and free from serious side-effects upon the patients.

In the present account the study has been extended by the addition of a further 42 patients, of whom half were treated and half served as controls. The trial has been terminated because the total of 75 patients has seemed to provide adequate information concerning the safety, potency, and usefulness of the preparation.

It is not proposed to recapitulate in detail the design of the trial and the methods used. In all patients the onset of infarction was abrupt and could be accurately timed as occurring not more than 12 hours prior to the commencement of treatment in hospital. Criteria for acceptance were a strong clinical diagnosis of a fresh