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Research Article

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Partial Purification and Properties of a Plasminogen Activator from Human Erythrocytes

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ABSTRACT The lysis time of euglobulin clots made with whole blood (plasma and red cells) was very much shorter than that of clots made with plasma alone, indicating a fibrinolytic component in red cells. A plasminogen activator was found in the stroma-free hemolysate, and proteolytic activity was found in the stromal fraction. The plasminogen activator, purified by using diethylaminoethyl-cellulose (DEAE-cellulose) in a batch procedure followed by column chromatography, was called erythrokinase (EK). On preliminary characterization, EK appears to activate human and bovine plasminogen in a manner similar to urokinase (UK), as determined by fibrinolytic and caseinolytic assays. The two enzymes can be separated by DEAE chromatography and acrylamide-gel electrophoresis, however, and they hydrolyze acetyl-L-lysine methyl ester and benzoyl arginine methyl ester at different rates.

INTRODUCTION

The plasminogen activators streptokinase (SK), from the hemolytic streptococcus, and urokinase (UK), from human urine, have been used under controlled conditions to produce experimental thrombolysis in man (1, 2). However, it has been shown that thrombolysis *in vivo* is influenced by the amounts of the various fibrinolytic components in plasma (3), other body fluids (4), various tissues (5), and by the components of the thrombus itself: fibrin stabilizing factor, fibrin, platelets (6), white blood cells (7), and erythrocytes (8).

Although large numbers of erythrocytes are usually enmeshed in thrombi, there has been relatively limited

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research on the fibrinolytic components contained in the red cell, or on the possible physiologic role of red cells in thrombolysis.

Kolmen, Guest, and Celander (8) showed that erythrocytes may absorb UK and protect it from plasma inhibitors. Kunzer and Haberhausen (9) found "plasmin-like activity" in the red cell stroma. Back et al. (10) extracted a fibrinolytic "potentiating agent" from human erythrocytes which appeared to increase the lytic potential of a given dose of UK or UK-activated plasminogen on human blood clots or thrombi. Tymiński and Czestochowska found a fibrinolytic, plasminogen-like proenzyme and traces of an activating substance in the hemolysate (11).

This communication is concerned with the isolation and characterization of a new factor present in human erythrocytes, a plasminogen activator of the urokinase type which will be called erythrokinase (EK). The new factor will also be physically and chemically distinguished from urokinase (UK).

METHODS

Sodium phosphate buffer (0.003 moles/liter, pH 7.0) was used unless noted otherwise. Diethylaminoethyl-cellulose (DEAE),¹ 1 mEq/g, was recycled for use by the method of Sober, Gutter, Wyckoff, and Peterson (12) and equilibrated in phosphate buffer. The columns were 64 × 2.5 cm, and the flow rate was set at 50 ml/hr with a positive-displacement pump.²

EK and UK were subjected to disc electrophoresis on acrylamide gel, according to the method of Ornstein (13) and Davis (14) in a Canalco³ disc electrophoresis apparatus. Continuous systems were run at pH 7.2 and pH 2.3, as well as discontinuous systems described by Williams and Reisfeld (15) at pH 4.5 and pH 9.0, for 2 hr at 5 ma per tube, using 7% acrylamide gel. The gels were stained with

¹ Whatman DEAE-Cellulose, H. Reeve Angel & Co., Inc., Clifton, N. J.

² Sigmamotor Pump, Model TM-20, Sigmamotor Inc., Middleport, N. Y.

³ Canal Industries, Bethesda, Md.

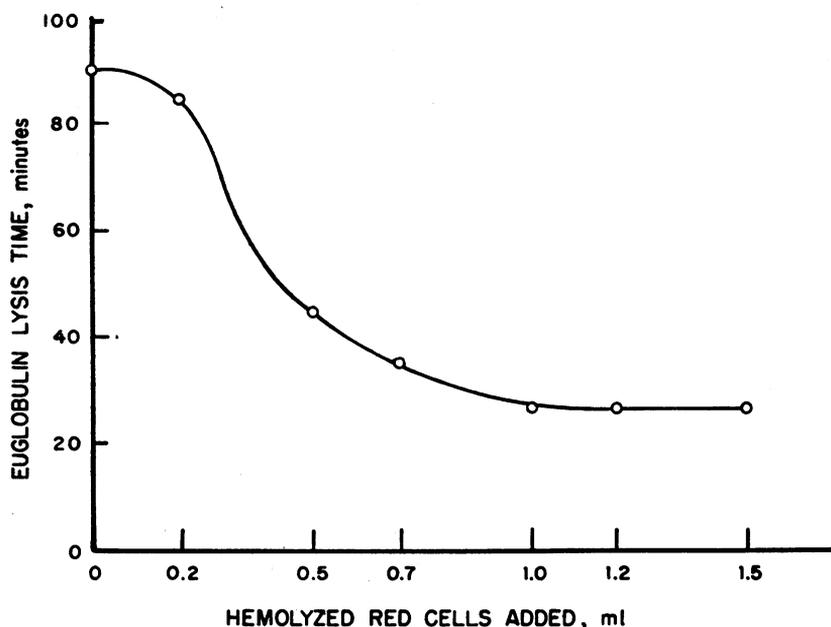


FIGURE 1 Effect of increasing amounts of red cells on plasma euglobulin lysis time. Varying amounts of washed, hemolyzed red cells from one subject were added to a constant amount of his plasma (0.7 ml), and the euglobulin precipitated from this mixture was reconstituted in saline phosphate buffer.

aniline black or cut with a Canaco lateral gel slicer into discs 1.0 mm thick. The discs were eluted for assay with 1 M NaCl and dialyzed against 0.01 M phosphate-buffered normal saline, pH 7.2.

Measurement of fibrinolytic activity. The clot lysis time was used to measure the fibrinolytic activity of euglobulin fractions from whole blood or plasma (16). The activity of column fractions was measured on heated and unheated bovine fibrin plates (17) to differentiate activator and plasmin. The activator activity of UK⁴ and partially purified EK was determined by a standard clot fibrinolytic assay system on intermediate- or high-purity soluble plasminogen (with <1% spontaneous plasmin activity) and a caseinolytic assay system (alpha casein) (16, 18). Protein determinations were carried out on these materials by the biuret procedure (19). Esterase activity of UK and EK was measured by hydrolysis of benzoyl arginine methyl ester (BAME)⁵ and acetyl-L-lysine methyl ester (ALMe)⁶ (20). The effect of different fibrinolytic inhibitors on EK and UK was also determined (16, 21). These inhibitors included: epsilon aminocaproic acid (EACA),⁷ Trasylol,⁸ Iniprol,⁹ and 4-aminomethylbenzoic acid.¹⁰

⁴ Kindly supplied by Abbott Laboratories, North Chicago, Ill., and Sterling-Winthrop Research Institute, a Division of Sterling Drug, Inc., Rensselaer, N. Y.

⁵ Mann Research Labs. Inc., New York.

⁶ Cyclo Chemical Co., Los Angeles, Calif.

⁷ Lederle Laboratories, Pearl River, N. Y.

⁸ Farbenfabriken Bayer AG, Leverkusen, Germany; U. S. distributor, FBA Pharmaceuticals Inc., New York.

⁹ Laboratoire Choay, Paris, France.

¹⁰ Sterling-Winthrop Research Institute, a Division of Sterling Drug, Inc., Rensselaer, N. Y.

Preparation of red cell hemolysate. Red cells were obtained from outdated human blood (over 3 wk old) collected by the American National Red Cross in acid-citrate dextrose (ACD), 435 ml blood plus 65.25 ml ACD (Formula A of the NIH). The cells were washed at least three times with an equal volume of cold isotonic saline (4°C), centrifuged at 10,000 g for 15 min in a refrigerated centrifuge after which the supernatant and leukocyte layer were discarded. The cells were hemolyzed by adding an equal volume of distilled water at 4°C and freeze-thawing five times in an acetone-dry ice mixture. The hemolysate was then diluted × 50 with 0.003 M phosphate buffer, pH 7.0. When 5% dextrose was used in a settling tank to aggregate and wash large volumes of red cells, the freeze-thawing step was omitted. These stroma-containing solutions will be called the original hemolysate.

RESULTS

The whole-blood euglobulin lysis time was previously found to be shorter than the plasma euglobulin lysis time at all levels of fibrinolytic activity (16). For example, the average lysis time for 33 normal men and women was 66 ± 6 min (SE) for whole-blood euglobulin and 162 ± 24 min (SE) for plasma euglobulin. In view of this marked difference in activity, varying amounts of hemolyzed red cells were added to a constant volume of plasma. The euglobulin fraction was prepared for each dilution, and reconstituted to a final volume of 1.0 ml. Fig. 1 shows that lysis was enhanced by increasing amounts of hemolyzed red cells.

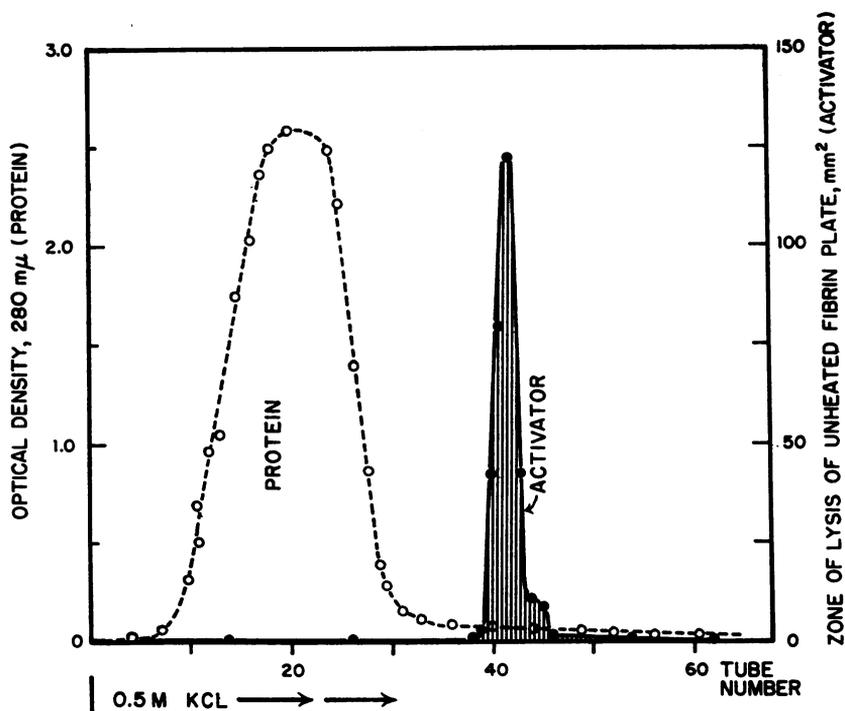


FIGURE 2 Separation of erythrocyte activator from hemolysate by DEAE cellulose chromatography. Original hemolysate was adsorbed on DEAE by batch methods and washed with 0.003 M phosphate buffer, pH 7.0, to remove the hemoglobin and the plasmin-like activity. This DEAE, with adsorbed activator, was placed on top of a previously prepared DEAE column and eluted with 0.5 M KCl.

In view of this observation, attempts were made to isolate the erythrocyte factor responsible for the enhanced fibrinolytic activity. The intact washed erythrocytes had little or no fibrinolytic activity when tested by the sensitive fibrin plate method. However, when the stroma was separated from the original hemolysate by centrifugation in a preparative ultracentrifuge²¹ at 150,000 *g*, fibrin plate assays indicated proteolytic activity with little activator in the stroma, 80 mm² (SE ±9) on heated bovine fibrin plates and 84 mm² (SE ±9) on unheated plates. Activator with little proteolytic activity was found in the stroma-free hemolysate; the zone of lysis was 66 mm² (SE ±5) on unheated bovine fibrin plates and 9 mm² (SE ±5) on the heated plates.

Purification of EK. DEAE-cellulose was used in a batch procedure for preliminary separation of EK from hemoglobin in the original hemolysate, since Hennessey, Waltersdorff, Huennekens, and Gabrio (22) reported that hemoglobin was not adsorbed on DEAE-cellulose. A 5% suspension of DEAE in phosphate buffer was added to the original hemolysate in a 1:150 ratio (v/v), the mixture was stirred at 4°C for 10 min, and the he-

molglobin-containing supernatant was decanted. Additional hemoglobin and the plasmin-like proteolytic activity were removed by washing the DEAE-cellulose with phosphate buffer until the final wash was colorless. This washed DEAE-cellulose, with adsorbed activator, was placed on the top of a previously prepared DEAE column so that the added portion represented less than 10% of the total volume. EK was then eluted from the column with 0.5 M KCl, and 10 ml samples were collected. The optical density of each sample was determined at 280 mμ (Fig. 2). (When the original hemolysate was placed directly on the column, proteolytic activity was noted in the main protein area which was found to be clearly separated from activator activity.) Material from the activator *peak* area, dialyzed against distilled water, lyophilized, and reconstituted in phosphate buffer, was used in all subsequent studies.

The EK preparation contained 750 CTA U²²/mg protein, representing at least 1000-fold purification over the original hemolysate. In contrast, the potency of highly purified urokinase preparations is approximately

²² Activity of the EK unit is expressed in similar terms to that of the CTA unit of UK, as recently defined by the Committee on Thrombolytic Agents (CTA) of the National Heart Institute (18).

²¹ Spinco model L Ultracentrifuge, Spinco Division, Beckman Instruments, Inc., Fullerton, Calif.

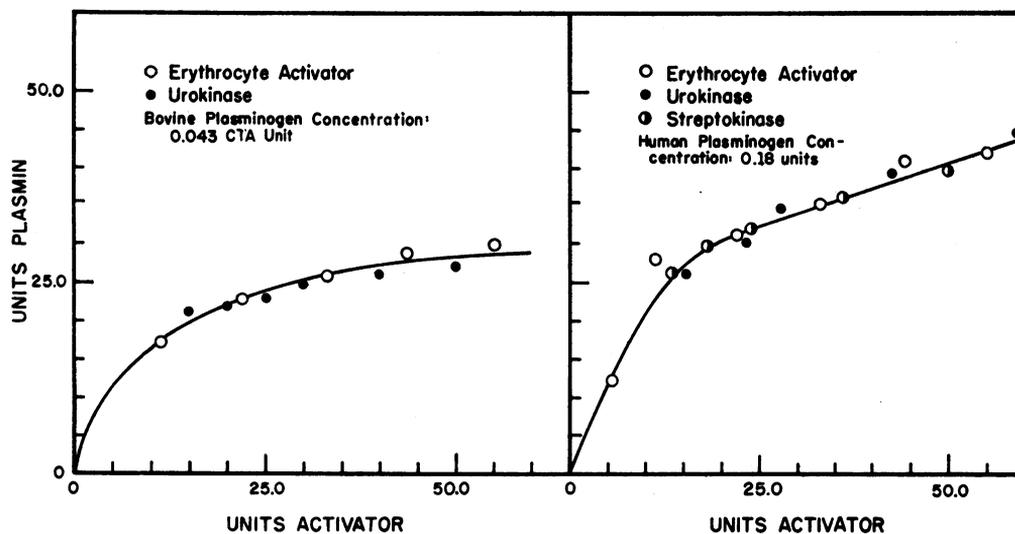


FIGURE 3 Comparative activity induced by equivalent fibrinolytic units of EK, UK, and SK. Number of fibrinolytic units of plasmin was calculated from standard curve of lysis time versus CTA units of plasmin.

50,000 CTA U/mg protein, and of pig heart activator, 18,000 CTA U/mg protein. Erythrokinase was prepared from fresh or outdated human red cells by the simple procedure described above. The average recovery from each of nine batches of 40 liters was 25 CTA U/liter of red cells, compared with the usual recovery of 800 CTA U of urokinase/liter of urine (23).

Properties of the activator. In the fibrinolytic assay, partially purified EK activated bovine plasminogen in the same way as did equivalent amounts of UK (Fig. 3). When tested with human plasminogen, EK, UK, and SK reacted similarly. This figure clearly shows that EK activates both bovine and human plasminogen. Caseinolytic assays for EK, performed with purified plasminogen, confirmed the role of EK as a plasminogen activator. Incubation of EK with alpha casein (but without plasminogen) did not result in casein hydrolysis. The activator activity of EK and UK was determined with purified plasminogen as primary

substrate and alpha casein as indicator substrate (Fig. 4); it was also compared with the fibrinolytic activity of each enzyme, when plasminogen was used as primary substrate and fibrinogen as indicator substrate. From these data, the activity of 1 U of EK was approximately the same as that of 1 CTA U of UK, whether measured by the caseinolytic or fibrinolytic assay, and their units of activity were therefore considered interchangeable.

The EK preparation was demonstrated to be free of proactivator and plasminogen inasmuch as bovine fibrin clots with added EK failed to lyse more rapidly with the addition of varying amounts of SK, i.e., 1 to 5000 modified Christensen units (16).

The effect of pH on denaturation of EK is shown in Fig. 5. EK was preincubated at 26°C for 60 min at various pH levels before being returned to neutral pH, and the residual activity was determined on fibrin plates. EK was found to be stable over a range of pH 6-8. Incubation at varying temperatures revealed its relative stability for 8 hr from -40° to +37°C (Fig. 6). Denaturation was significant at temperatures above 37°C.

EK was tested for thromboplastic or partial thromboplastic activity by a kaolin-activated partial thromboplastin time test (24). In this procedure, 0.1 ml of a kaolin suspension in normal saline was incubated with 0.1 ml of EK (750 CTA U/ml) and 0.1 ml of citrated, platelet-poor plasma at 37°C for 3 min before the addition of 0.1 ml of 0.025 M CaCl. Clotting times with 750 CTA U of added EK were similar to those of the

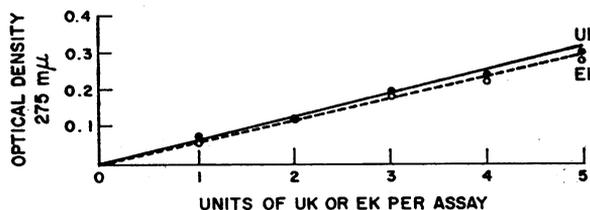


FIGURE 4 Caseinolytic assay of EK and UK (CTA units). Intermediate-purity plasminogen was used as the primary substrate.

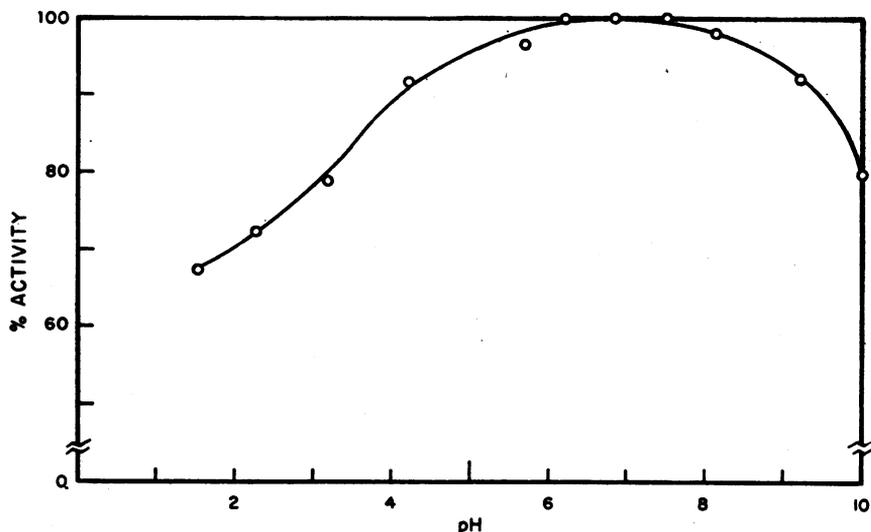


FIGURE 5 Effect of pH on denaturation of erythrocyte activator. pH of sample is maintained for 60 min at 26°C, and then adjusted to 7.5 before assay.

blank; thus, no clot-promoting or thromboplastic activity was demonstrable under these conditions.

Differentiation from urokinase. Erythrokinase and urokinase were clearly differentiated by various techniques, including acrylamide-gel electrophoresis (Fig. 7). As mentioned previously, the EK preparation made by DEAE chromatography contained about 750 CTA U/mg protein. It showed three bands on acrylamide-

gel electrophoresis; the EK activity in two of them (11,000 and 3800 U/mg protein, respectively) represented 55% of the total protein in the preparation. The third band contained none.

Erythrokinase and urokinase were also clearly differentiated through study of the hydrolysis of benzoyl arginine methyl ester. The rate of hydrolysis of BAME by these enzymes was independent of substrate con-

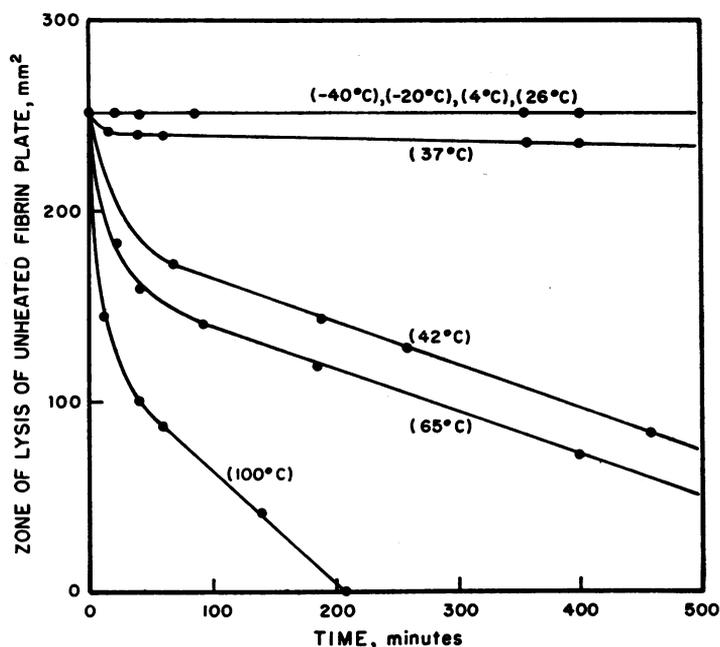


FIGURE 6 Effect of increasing temperature on denaturation of erythrocyte activator.

centration, indicating zero order kinetics, at or above 5×10^{-4} M BAME (20). Under these conditions, the reaction rate for EK was found to be approximately 2.8 times that for UK. Equivalent units of each enzyme were used over a range of 250–2500 CTA U at a BAME concentration of 1×10^{-3} M (Table I). Similarly, when data from hydrolysis of acetyl-L-lysine methyl ester were plotted by the Lineweaver and Burk method (25), differences were noted in the Michaelis constants (K_m) and maximum velocities (V_m), as shown in Fig. 8.

When EK isolated by the above procedure was mixed with UK and chromatographed on DEAE-cellulose, peaks of the two enzymes were clearly separated (Fig. 9). Control runs with UK and EK, respectively, confirmed the individual identities of the peaks.

EK and UK must have a very similar mode of action, however, since both enzymes were inhibited by almost the same concentrations of all but one of the fibrinolytic inhibitors tested, the exception being 4-aminomethylbenzoic acid (Table II).

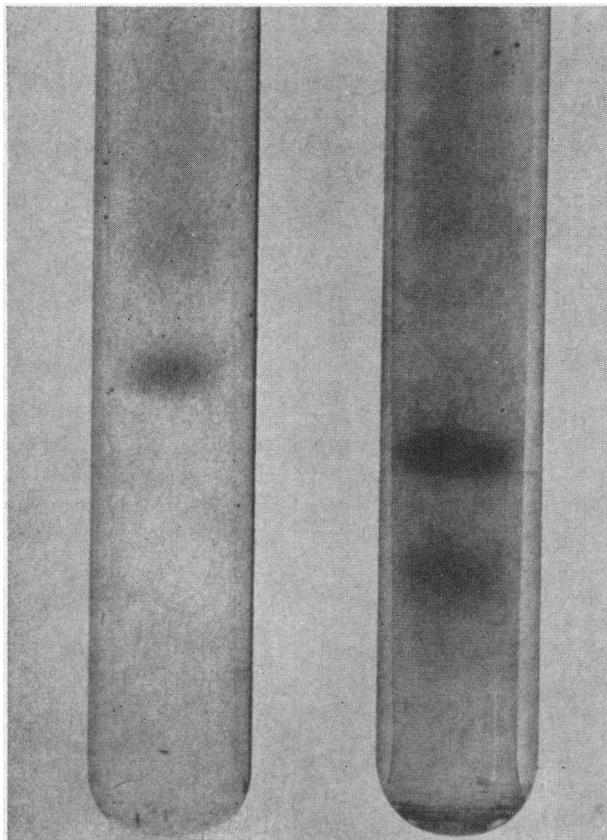


FIGURE 7 Acrylamide gel electrophoresis: samples of EK (right) and UK using a single gel (7%) system. The samples (2% protein) were placed on top of the gel and run in a continuous buffer system with 0.05 M glycine HCl buffer at pH 2.3 for 2 hr at 5 v/cm.

TABLE I
Hydrolysis of Benzoyl Arginine Methyl ester (BAME)
by Equivalent CTA Units of Erythrokinase (EK)
and Urokinase (UK)

Enzyme activity	Erythrokinase	Urokinase	Ratio V_{EK}/V_{UK}
CTA units	mmoles BAME/min per unit $\times 10^{-3}$		
2500	—	2.46	—
1250	7.10	2.64	2.69
750	6.50	2.22	2.92
500	6.54	2.28	2.87
250	7.24	2.44	2.96

DISCUSSION

EK, an enzyme activator of plasminogen which is found in stroma-free hemolysate, is stable under physiologic conditions of pH and temperature. It is readily distinguished from the proteolytic activity in red cells noted by others (26). Since the partially purified erythrocyte activator contains no proactivator, as did the preparation of Tymiński and Czeszochowska (11), or plasmin activity, as did the lytic agent reported by Kunzer and Haberhausen (9), it may be somewhat similar to the activator observed by the Polish investigators in the "hemoglobin-free" red cell membranes. In the present study, however, activator was found in the stroma-free hemolysate and proteolysis with little or no activator was found in the washed, ultracentrifuged stroma. EK differs markedly from the fibrinolytic potentiating factor (FP) described by Back et al. (10) which is dialyzable and heat-stable and does not activate plasminogen itself but requires activator for its potentiating activity.

EK, like UK and other "tissue activators" (27), activates both bovine and human plasminogen. In contrast, EK is localized within the red cells and hydrolyzes BAME and ALMe more rapidly than does UK. Further-

TABLE II
50% Inhibition of 50 CTA Units of Erythrocyte and Urokinase Activators, Respectively, in a Plasminogen-Rich Standard Clot System (See Methods)

Inhibitor	Concentration required for 50% inhibition of:	
	Erythrocyte activator	Urokinase activator
	mg/ml	mg/ml
EACA	9.8×10^{-3}	4.1×10^{-3}
4-Aminomethylbenzoic acid	1.1×10^{-2}	2.3×10^{-3}
TrasyloI	6.7×10^{-4}	1.05×10^{-3}
Iniprol	8.5×10^{-5}	1.08×10^{-4}

EACA = epsilon aminocaproic acid.

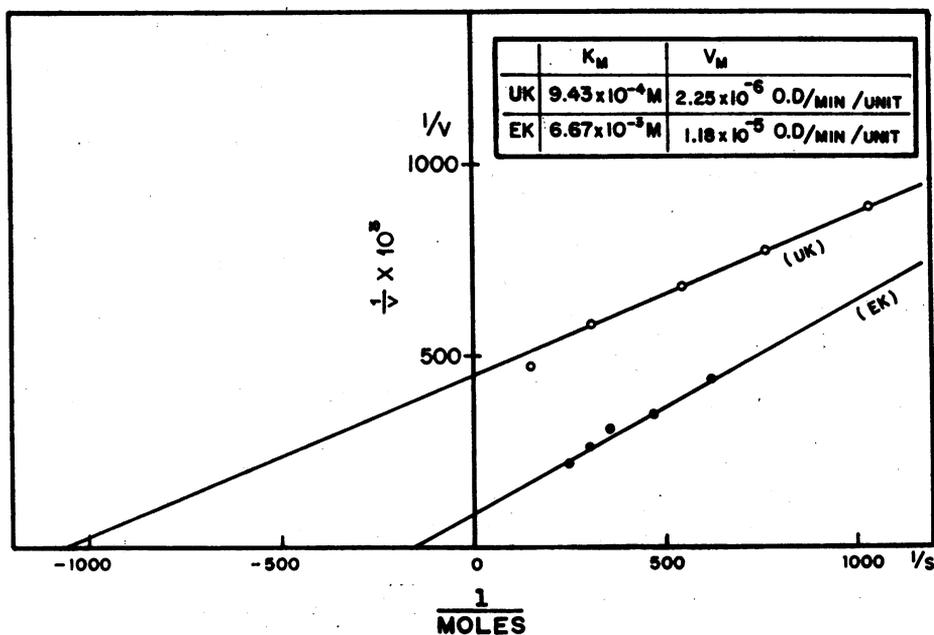


FIGURE 8 Lineweaver-Burk plot showing hydrolysis of ALMe by urokinase (UK) and erythrokinase (EK). On ordinate: reciprocal of velocity (V) measured as change in OD/min/CTA unit. On abscissa: reciprocal of concentration (in moles) of substrate ALMe.

more, when the two enzymes are run on acrylamide-gel electrophoresis, their mobility differs sharply, and EK is eluted separately from UK on DEAE chromatography. No attempts were made to carry out biochemical and

biophysical studies comparing EK and UK, which are freely soluble at neutral pH, with "tissue activators," which require 2 M potassium isothiocyanate at acid pH for solubilization (28).

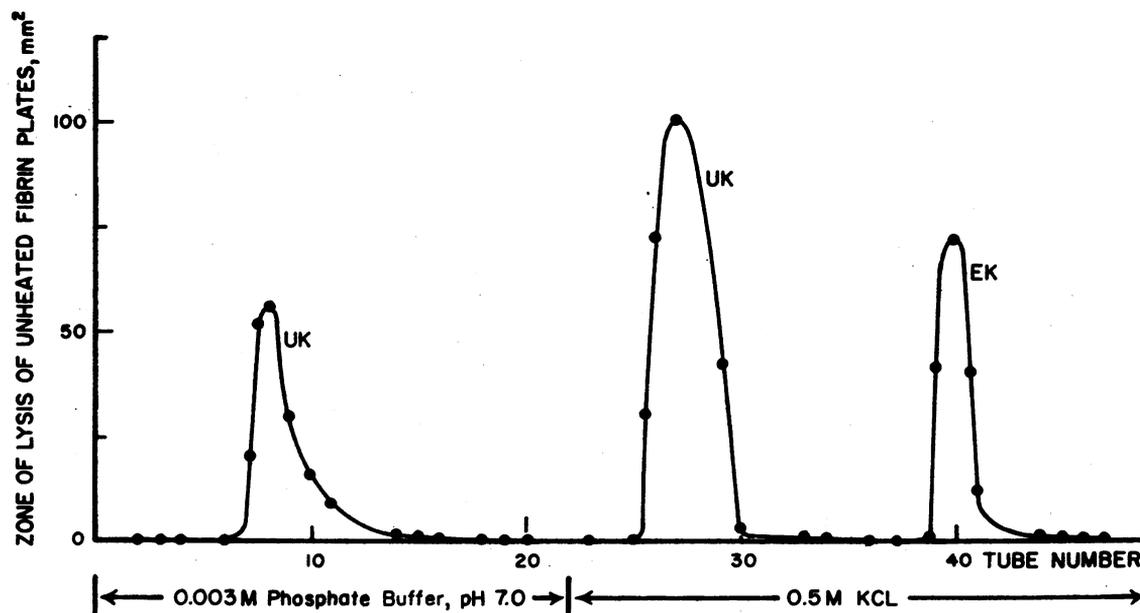


FIGURE 9 Separation (by DEAE cellulose chromatography) of purified urokinase (UK) from partially purified erythrokinase (EK).

The specific activity of EK prepared by DEAE chromatography was only about 750 CTA U/mg protein. However, on acrylamide-gel electrophoresis, one of the three bands had a specific activity of 11,000 CTA U; another had 3800 CTA U, and the third (representing 45% of the total protein in the preparation) had none. Since the amount of extraneous protein seems relatively small, a higher degree of purification would be expected unless a considerable amount of EK becomes denatured during preparation. In any event, the potential purification and specific activity are not yet known.

Erythrocytes may possibly play a role in physiologic thrombolysis. Hemolysis has been observed by electron microscopy during hemostasis after transection of a small mesenteric arteriole in a guinea pig (29), and nearly 15 mg of hemoglobin/100 ml serum was found in human blood coagulated for 30 min in siliconed or glass test tubes (30). However, the relatively small amounts of activator released during hemolysis, and the tendency for a newly forming thrombus to adsorb activators, suggest that any thrombolytic effect would probably be localized in the thrombus.

ACKNOWLEDGMENTS

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