Partial Purification of Urinary Erythropoietin.* (24041)

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Erythropoietic stimulating activity has been detected in urines of anemic rabbits(1) and some human subjects with low hemoglobin levels (below 6 g%)(2-6). Urinary erythropoietic substance(s) when administered to rats may evoke increases in total numbers of circulating erythrocytes(6) as well as in total circulating red cell volume(5). Reticulocytosis(2,5,6), marrow erythroid hyperplasia (2,6) and enhanced incorporation of Fe^{59} into the circulating erythrocytes(4) have also been observed. A shorter assay, involving reticulocyte and hematocrit determinations, was employed in this study to expedite screening of large numbers of chemical fractions. A significant increase in reticulocyte percentages unaccompanied by fall in hematocrit after 5 days treatment constituted a positive indication of presence of erythropoietic stimulating activity. From the work of Rambach *et al.* (7), and our own experiences indicating a positive correlation between toluidine blue metachromasia and presence of erythropoietic stimulating activity in plasma filtrates(8), it appeared possible that plasma erythropoietin is a mucoprotein or a moiety associated with mucoprotein. Findings of elevated mucoprotein levels in both serum of anemic rabbits (9) and in urine of rabbits rendered anemic by repeated bleedings(10) present further circumstantial support for this possibility. Urinary gonadotrophin is a substance(s) known to have chemical properties of a mucoprotein, and ultrafiltration technics for concentration of urinary gonadotrophin(11) have been recently applied(5) for concentration of urinary erythropoietic activity. We have utilized a modification of another urinary gonadotrophin procedure, namely the kaolin adsorption method(12), in an attempt to purify urinary erythropoietin.

Materials and methods. Urine for this fractionation was obtained from a 5-year-old boy with Cooley's anemia.† His hemoglobin level at time of urine collection was 4.5 g%. Mature Long-Evans female rats weighing 165 to 200 g (average 185 g) and maintained on a diet of Purina Chow *ad lib.* were employed for assay of filtered untreated urine. Four rats received subcutaneous injections of 1 ml daily and 4 received 3 ml daily. Reticulocyte counts were done using Brecher's method(13) prior to the injection period and on the day following fifth injections. Microcapillary hematocrit determinations were performed in duplicate at the same times. The mean increases in these 2 parameters minus mean changes in 4 untreated controls are shown in Table I.‡ Acid washed American Standard kaolin (Fisher K-5) was used for adsorption of the urinary factor. Preliminary studies demonstrated that the amount of active material adsorbed did not vary much over pH range of 4.3 to 6.2 although pH 4.8 appeared to give slightly better results than the others. Four and a half g of kaolin was added, with stirring, to 300 ml of urine. The pH was lowered to 4.8 by addition of 2.6 ml of 1N HCl. The suspension was equilibrated for 24 hours at 5°C. The kaolin was removed by centrifugation and the supernatant fluid equilibrated with a second 4.5 g of kaolin as before. Buffers were prepared by addition of 1M acetic acid to 1M NH_4OH until solutions with pH 8, 9, and 10 were obtained. The first kaolin precipitate was then suspended, by mixing several times with a pipette, in 20 ml of cold pH 8 ammonium acetate buffer and spun

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† We wish to thank Dr. Harold Fink, Coney Island Hospital, Brooklyn, N. Y., for making available urine samples from this patient.

‡ All mean control reticulocyte and hematocrit values were essentially unchanged throughout experiment. Mean reticulocyte values varied no more than .2/100 RBC and mean hematocrits varied less than 1.1% in packed cell volume.

down again. The 20 ml of supernatant fluid was placed in the freezer. This procedure was repeated twice using pH 8 buffer and then performed 3 times with each of the pH 9, pH 10, 1M NH_4OH, and 2M NH_4OH eluting solutions. Sixty ml of each eluate was therefore collected and frozen separately. The kaolin precipitate from the second equilibration was subsequently eluted twice with each buffer, first with 20 ml and then with 5 ml. This, added to the rest, yielded 85 ml of each eluate solution. The eluates were placed separately in Visking tubing and dialyzed at 5°C for 24 hours against 0.9% saline, then 24 hours against a 0.1M phosphate buffer (pH 6.8), and finally 48 hours against 0.9% saline again. All eluates were essentially neutral reacting to pH paper, reading about pH 6.8. Six groups of female rats averaging 185 to 195 g group were used to assay the eluates. Four rats were employed in each group, and the group mean reticulocyte and hematocrit values were carefully matched before treatment. Three ml daily subcutaneous injections were given to each rat for 5 days. Reticulocyte and hematocrit values were again determined on the sixth day. The control group received similar amounts of 0.9% saline. All determinations were performed on the same days. Mean increases in peripheral parameters of treated groups minus mean changes in the control group are shown in Fig. 1.‡ The nondialyzable solids content of the eluates was determined by dialysis against distilled water for 36 hours and evaporation to dryness, carefully with an infra-red lamp, of 4 ml aliquots of the dialysand in aluminum weighing pans. Toluidine blue metachromasia was tested for by adding 0.05 ml of an 0.025% solution of toluidine blue O dissolved in .1M KH_2PO_4 to 0.2 ml of the solution under observation. Ultraviolet absorption spectra were taken of the eluates in a Beckman Model DU Spectrophotometer, using silica cells with 1 cm light path. An ultracentrifuge analysis was performed by Dr. Ralph Heimer on an aliquot of the 1M NH_4OH eluate concentrated 4X by dialysis against 40% polyvinyl pyrrolidone in physiological saline. This solution was spun 2 hours in a Spinco Model E analytical ultracentrifuge at 145,000 X gravity. Paper strip electrophoresis of 5 μl of the 1M eluate or of the 4X concentrate of this eluate was conducted on Whatman 3MM strips in a Spinco Model R electrophoresis cell at a constant current of 6 milliamps and 85 volts. Veronal buffer pH 8.6, ionic strength 0.075 was employed and 16 hours were allowed for migration. The strips were then dried in an oven and stained with bromphenol blue. Statistical methods used were those described by Davies(14).

TABLE I. Bioassay of Whole Anemic Human Urine.

Dose, ml/day	Response (mean increase minus control change ± S.E.) Reticulocytes/100 RBC	Hematocrit, % packed cells
1	1.1 ± .5*	.7 ± .9
3	3.1 ± .9†	2.0 ± 1.1

* Increase over pre-treatment level—P <.05.
† *Idem* —P <.01.

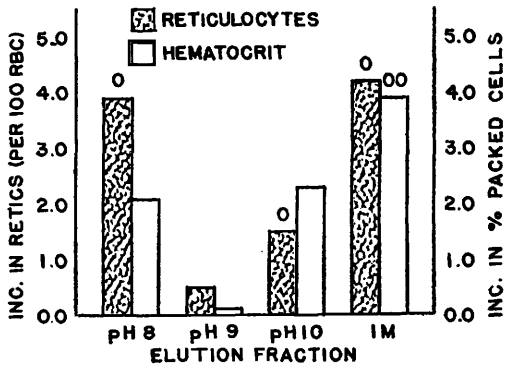

FIG. 1. Erythropoietic activity of eluates of urinary substance adsorbed on kaolin at pH 4.8. Significance of increase over pre-treatment levels, 0 = P <.01, 00 = P <.02.

Results. The activity recovered in different eluates from kaolin precipitates can be seen in Fig. 1. The principal active fractions were those obtained by elution at pH 8 and by elution with 1M NH_4OH. The 2M NH_4OH eluate evoked a decrease in hematocrit, hence its activity could not be determined. Since proportionality between dose of urine and reticulocyte response appears to be linear in the concentration ranges employed (Table I) it may be calculated that 36% of the original activity was recovered at pH 8, 5% at pH 9, 14% at pH 10, and 39% with 1M NH_4OH. The total recovery for all fractions was thus

TABLE II. Purity of Urinary Erythropoietin Eluted from Kaolin.

Fraction	Solid content, mg/ml	% relative activity/mg	Purification
Original urine	39.50	2.5	1
Idem (dialyzed)	1.70	59.0	24×
pH 8 eluate	3.40	37.1	15×
1M NH_4OH eluate	0.23	587.0	230×

94%. The % relative activity was obtained by dividing reticulocyte response to 3 ml daily injections of a given fraction (Fig. 1) by the response to 3 ml daily injections of starting material (Table I) and multiplying by 100. When this figure is divided by the solid content of the solution, the % relative activity/mg is obtained. Dividing % relative activity/mg of a given fraction by that of starting material provided an index of purification (Table II). Thus, dialysis alone produced a 24-fold purification while the 1M eluate represented a 230-fold purification or approximately a 10-fold purification of the nondialyzable material.

The height of the absorption peak at 280 mμ roughly paralleled the erythropoietic activity of the fractions (Fig. 2). The most potent fraction, the 1M NH_4OH eluate absorbed considerable light at this wave length. This fraction also produced a pronounced metachromatic reaction with toluidine blue. No metachromasia was produced by the original urine or by fractions eluted with the less basic buffers. When concentrated 4 times by dialysis against 40% polyvinyl pyrrolidone and subjected to a relative centrifugal force of 145,000 X g for 2 hours there was very little displacement of the boundary, and no sharp peaks appeared. The electrophoretic pattern obtained from 5 μl of the 1M eluate revealed very little migration of a weakly staining spot near the origin of the paper strip.

Discussion. The use of kaolin for adsorption of urinary erythropoietin has enabled recovery in the 1M NH_4OH eluate of 39% of the original activity at a purity 230 times that of raw urine. This purified preparation produced a strong metachromatic reaction with toluidine blue. A similar correlation has been reported(8) between metachromasia and erythropoietic stimulating activity in boiled plasma filtrates from anemic rabbits. Failure of the original urine and the pH 8 eluate to display metachromasia may have resulted from the presence of metachromasia inhibitors. Indeed, we have found that heparin added to anemic human urine is incapable of displaying its usual pink metachromatic reaction. Presence of metachromatic properties and an absorption peak at 280 mμ in our purest fraction is not inconsistent with the suggestion that erythropoietin may be a mucoprotein or an associated substance(7). However, urinary erythropoietin does not possess any material that migrates in the paper electrophoretic system as far as alpha globulins found in anemic rabbit plasma(7). The position on the paper strip of our preparation is more like that assumed by Marcotte-Boy's fraction X(10).

Others(15-17) have suggested that erythropoietin may be a lipid and this possibility cannot be precluded. However, unlike the plasma factor of Linman and Bethell(15), urinary erythropoietin can evoke significant increases in hematocrit(6) or in circulating red cell volume(5). It would be of interest to test the combined effects of these 2 preparations.

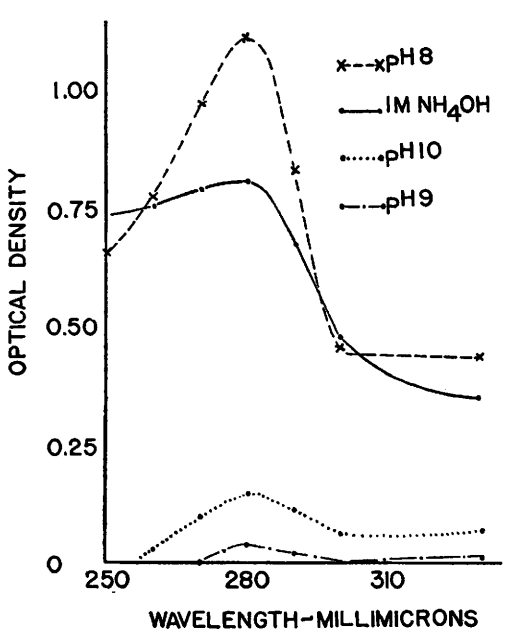

FIG. 2. Ultraviolet absorption spectra of the eluates.

Work is in progress to determine the smallest biologically active moiety in urine, its chemical nature, and whether its primary site of action is bone marrow or some other tissue or organ.

Summary. Urinary erythropoietic factor has been partially purified by adsorption on kaolin followed by serial elution with ammonium acetate buffers of increasing pH and then with 1M NH_4OH. Using this procedure, a 39% yield with a 230 fold purification of the active factor has been obtained. The purified preparation of the active factor produces metachromasia with toluidine blue, has an absorption peak at 280 mμ, and migrates very slowly on paper strips in an electrophoretic apparatus. The similarity and differences between urinary and plasma erythropoietin are discussed.

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Influence of Cortisone, Medrol, and Aristocort Diacetate on Antitryptic Activity of Rat Serum. (24042)

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The view has been expressed that an intimate physiological relationship exists within the circulating blood between fibrin formation and dissolution (fibrinolytic) mechanisms and the integrity of the blood vessel wall(1,2). Previous work(3,4) has presented evidence that the pituitary-adrenal cortex axis exerts a controlling influence on the fibrinolytic (plasmin-antiplasmin) system in the blood. In continuation of studies(5) on the influence of certain modified corticosteroids, more potent than cortisone, on antitryptic activity of rat serum, Medrol (Δ^1-6-α methylcortisol) and Aristocort diacetate (16α-hydroxy-9α-fluorocortisol-16α, 21 diacetate) were compared with cortisone in normal, hypophysectomized and adrenalectomized rats.

Materials and methods. Male Sprague-Dawley rats weighing 150-200 g were used. Normal and hypophysectomized animals were allowed Purina Laboratory chow and water *ad libitum,* while adrenalectomized rats were fed Purina Laboratory chow and 0.9% saline *ad libitum.* All administrations were given intramuscularly; controls received either no injections, or a volume of 0.9% saline equal to that used in the steroid injections. Adrenalectomy and hypophysectomy were performed