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Erythropoietic Activity in Human Urine Concentrate.* (29361)

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Urine, concentrated by flash evaporation, has been used by several investigators to stimulate erythropoiesis in assay animals(1, 2,3). The purpose of this work is to characterize the activity of erythropoietin prepared from urine by flash evaporation and ethanol precipitation.

Materials and methods. All reagents used for preparative and analytical procedures were of reagent grade except that the ethyl alcohol which was U.S.P. Glucagon from Eli Lilly Co. and Thytropar from Armour Pharmaceutical Co. were used to characterize dialyzing membranes.

Urines, collected from patients with refractory anemia(2), were dialyzed against distilled water and then concentrated 10 to 20 times by flash evaporation at 37°C with a continuous flow apparatus. The dialyzing tubing was 1⅞ inch (inflated diameter) regenerated cellulose. The sediment from the urine concentrate was removed by centrifugation.

The alcohol precipitation of proteins was under controlled conditions as recommended by Cohn(4) and adapted by Pearsall and Chanutin(5). The urine concentrate was adjusted to pH 5.0 with an acetate buffer, and fractions were obtained at concentrations of 50 and 80 volumes per cent ethanol and at varying ionic strengths. All precipitates were dissolved in distilled water, dialyzed against distilled water, and assayed for erythropoietic activity.

Dialysis experiments were done on urine concentrates using two techniques. Ten to twenty ml aliquots of urine concentrate were dialyzed to equilibrium against equal volumes of distilled water for a period of 4-7 days at 2°C. The dialyzate was stirred thoroughly several times during each day. Erythropoietic activity was measured on the urine concentrate, the dialyzand and the dialyzate. The second technique differed in

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TABLE I. Effect of Ionic Strength on Erythropoietic Activity in Ethanol Fractions from Urine Concentrates.

Run	Initial ionic strength	Ethanol 50%		Ethanol 80%	
		Activity C.S. units*	Specific activity units†	Activity C.S. units*	Specific activity units†
1	.20	.49 ± .13	.08 ± .02	.49 ± .04	.06 ± .01
2	.30	.74 ± .16	.15 ± .03	.72 ± .06	.15 ± .01
3	.50	.44 ± .02	.14 ± .01	1.27 ± .11	.22 ± .02
4	.90	.22 ± .10	.04 ± .02	2.13 ± .07	.43 ± .01

Urine concentrate activity: 1.58 ± .24 C.S. units.*
Urine concentrate specific activity: .06 ± .01 units.†
Plasma erythropoietin control: 1.40 ± .16 units.*

* C.S. units with standard deviation of mean, % Fe^{59} incorporation in RBC of starved rats minus incorporation in saline injected rats. 1 unit = 20% Fe^{59}.
† Activity per mg of nitrogen injected.

that the dialyzate (10 × volume) was changed each day, and no erythropoietic activity measurements were made on the dialyzate. The membrane used for dialysis experiments was 22/32 Visking casing.

A modification of the method of Fried, Plzak, Jacobson, and Goldwasser was used as an indirect assay for erythropoietin(6). At least 4 starved rats were used in each assay. Injections were made intraperitoneally. The per cent of the injected Fe^{59} incorporated into the red blood cells was measured. The activity units were those defined by Keighley *et al*(7). One C.S. unit (cooperative study) of erythropoietin is defined as 20% of Fe^{59} incorporation in RBC of starved rats. The per cent Fe^{59} incorporation in RBC of the control animals injected with saline was subtracted as recommended. The specific activity was calculated as the C.S. units per mg of nitrogen injected. A plasma erythropoietin control, obtained from anemic patients(2), was run with each assay. The plasma control had been compared previously to sheep plasma erythropoietin.§ This procedure was used due to the limited supply of sheep plasma erythropoietin.

Results. The data obtained on urine that was concentrated by flash evaporation and fractionated with ethanol are shown in Table I. The fractions were obtained at ethanol concentrations of 50 and 80 volumes per cent, at pH 5.0, and by varying the ionic strength with sodium chloride. Specific activities ranged from 0.04-0.43 C.S. units per mg of nitrogen. As ionic strength increased, the bulk of erythropoietic activity appeared in the 80% ethanol fraction, and the specific activity increased.

Data from the assay of the dialyzates and dialyzands of active urine concentrates are given in Table II. The erythropoietic activities of the dialyzates were 2-4 times the saline control levels during equilibrium dialysis. The erythropoietic activities of the dialyzands were reduced to near the saline control levels by exhaustive dialysis. There were several equilibrium dialysis experiments in which no activity was detected in the dialyzates. The membrane used, during equilibrium and exhaustive dialysis experiments, did not allow any thyrotropic hormone (M.W. 10,000) to escape in 4 days. Glucagon (M.W. 3,485) dialyzed almost to equilibrium in 4 days.

Discussion. The combination of flash evaporation and high ionic strength denatured some of the proteins in the ethanol fractionation experiments However, the erythropoietic specific activity increased, and erythropoietin was relatively stable under the conditions described. As ionic strength increased, erythropoietin became more soluble in aqueous ethanol, and more erythropoietic activity shifted to the 80% ethanol fraction (Table I). Lowy and Borsook precipitated urinary erythropoietin at a lower ionic strength and found the erythropoietic activity to vary within precipitates obtained at the same ethanol concentration, but in most instances

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TABLE II. Dialysis Studies of Erythropoietic Activity in Urine Concentrates.

Exp	Days dialysis	Urine concentrate C.S. units*	Dialyzand C.S. units*	Dialyzate C.S. units*
		Equilibrium dialysis		
1	6	2.01 ± .27	.99 ± .40	.40 ± .33
2	4	1.63 ± .23	.99 ± .40	.67 ± .22
3	6	1.90 ± .19	1.47 ± .19	.31 ± .02
4	7	1.58 ± .24	1.64 ± .35	.70 ± .10
		Exhaustive dialysis		
1	4	1.63 ± .23	.20 ± .06	
2	3	.79 ± .20	.12 ± .07	
3	6	.79 ± .20	.09 ± .10	

Plasma erythropoietin control: 1.11 ± .21 units.* 1 unit = 20% Fe^{59}.

* C.S. units with standard deviation of mean, % Fe^{59} incorporation in RBC of started rats minus incorporation in saline injected rats.

the material with the highest specific activity was in the "60-70"% ethanol precipitates (8).

By increasing the ionic strength of the urine concentrate the total erythropoietic activity of the fractions increased. Since increasing the ionic strength with sodium chloride was the only variable in the experiment, sodium chloride apparently had a stabilizing effect on erythropoietin. The increase in erythropoietic activity can be seen by comparing the erythropoietic activity at high ionic strength with the activity at lower ionic strength and with the urine concentrate activity before fractionation.

The apparent stability of erythropoietin possibly was due also to the protective action of a carrier protein. The volume of the equilibrated dialyzate required to demonstrate activity was 2½ times that of the dialyzand. During equilibrium conditions less than one-fourth of the total erythropoietic activity was detected in the dialyzates. It would appear that most of the erythropoietin remaining in the dialyzand would be of such size and shape, or in combination with a carrier protein of such size and shape, as to be non-dialyzable through the membrane described. A carrier protein seemed more likely, since most of the activity was removed from the urine concentrate dialyzand by repeated changes of the dialyzate. Goldwasser, White, and Taylor(9), and Rambach, Alt, and Cooper(10) postulated the existence of a free and bound erythropoietin. The dialysis data added credence to this postulate. Goldwasser *et al* found that highly purified sheep erythropoietin was labile(9). It would seem that the carrier protein in human urine may be protective, since erythropoietin was relatively stable during flash evaporation, high ethanol concentration, and high ionic strength.

Most workers have reported erythropoietin to be nondialyzable(8,10,11). However, others have found that it is possible to dialyze erythropoietically active material through some membranes(12,13,14). Possibly the erratic results were due to variations in dialyzing membranes as demonstrated by Craig, Konigsberg, Stracher, and King(15).

Summary. Increased erythropoietic specific activities remained in urine which had been concentrated by flash evaporation, adjusted to high ionic strength, and precipitated at high ethanol concentration. The apparent stability of erythropoietin was attributed to a protective carrier relatively stable under the conditions described. Erythropoietic activity from urine concentrate was detected in the dialyzate from a membrane which retained thyrotropic hormone and allowed glucagon to escape. The dialysis data added credence to the postulate of a free and bound erythropoietin.

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"Auer Colitis" in Rabbits Induced by Intrarectal Antigen.* (29362)

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In 1920, Auer(1) produced a severe inflammation at the site of a previously induced, non-specific irritation, by injection of a large dose of the specific antigen to a sensitized animal. Previous studies from this laboratory have demonstrated an "Auer" inflammatory reaction in the distal colon ("colitis") of rabbits sensitized to crystalline egg albumin, after prior mild irritation of the bowel with dilute formalin; followed by intravenous or intraperitoneal administration of the specific antigen(2). Histologically, the Auer colitis was characterized by superficial ulceration and hemorrhage, infiltration with polymorphonuclear leukocytes, plasma cells and lymphocytes. Circumstantial evidence suggested the implication of a specific antigen-antibody reaction, facilitated by increased capillary permeability, induced by the exposure to mild formalin. Kraft and his associates(3) recently have provided direct evidence of the antigen-antibody nature of the Auer reaction in the colon. By immunofluorescent techniques, localization of the specific antigen and antibody was demonstrated only at the site of the "colitis" and not in the uninvolved colon or in the colon of sensitized and non-sensitized controls.

The present study was undertaken to determine if direct intrarectal instillation of the challenging antigen also would initiate an "Auer colitis."

Methods and materials. Sensitization. Adult New Zealand white rabbits, weighing 2 to 3 kg, and maintained on a diet of Purina Rabbit chow and water, were used exclusively. Sensitization was accomplished with 5 times crystallized egg albumin (Pentex) as the antigen, by the multiportal method described by Wissler *et al*(4). One ml of a 2% solution was injected into each of 5 portals (intravenous, intramuscular, intraperitoneal, subcutaneous and intradermal) daily, for a total of 100 mg. This schedule was repeated 3 days each week for 3 successive weeks. Occasionally, during the final week of sensitization the intradermal injections produced positive Arthus reactions; and sensitization was discontinued in these ani-

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