Concentration of Highly Potent Erythropoietic Activity from Urine of Anemic Patients.* (23533)

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Since the demonstration by Reissmann that erythropoiesis is under humoral control(1) the search for the site of production of this factor and for an adequate source from which erythropoietic hormone could be isolated has been the subject of much investigation. Erythropoietic activity has been found in a variety of body fluids, including plasma(2-4), urine (5,6), and milk(7), and erythropoietically active material has been isolated from the anterior pituitary(8). However, such activity has been demonstrated in body fluids primarily following some procedure which has decreased the oxygen-delivering ability of the blood (phenylhydrazine(2), hemorrhage(3), hypoxia(9), etc.), in disease states(4), or following administration of cobalt(10); from such conditions it has not been possible to isolate active fractions in sufficient quantity for adequate chemical and biological investigations.

In the course of screening a large number of patients with various hematological disorders for plasma erythropoietic titer, using the Fe^{59} incorporation in red cells as an assay (11,12), it was found that some patients with aplastic anemia showed a marked elevation in their titer. From the urine of some of these patients a highly potent erythropoietin has been extracted.

Material and methods. Normal female rats of the Long-Evans strain weighing between 150 and 200 g were used for the assay. They were fed a complete diet.† The rats were divided into groups of equal average weight at the beginning of any assay. Untreated urine or a concentrate of urine was injected subcutaneously once daily for 14 days. Control rats were injected with an equal volume of saline or urine from a normal subject. For comparison with the response obtained with urine concentrate, a group of rats was maintained continuously in a decompression chamber at a simulated altitude of 15,000 feet for 14 days. On the 12th day of injection, metabolic rates were determined with a closed-circuit multiple respiration apparatus, as described by Evans, *et al.*(13).‡ To measure I^{131} uptake by the thyroid, 0.5 μc of I^{131} was given intraperitoneally 16 hours prior to autopsy, and the activity of the thyroid determined after autopsy. Just prior to autopsy, blood volume and total circulating red cell volume were determined by the Fe^{59} labeled cell dilution method(14). At autopsy the adrenals, thyroid, ovaries, uterus, thymus, and spleen were dissected and weighed. The urine used in assays reported here was obtained from a Caucasian female of 11 years, who was first found to be anemic at the age of 1½ months and who has received transfusions monthly since the age of 3 months. Physical examination was essentially normal except for some enlargement of liver and spleen. Laboratory work has shown slightly hypochromic, normocytic erythrocytes, reticulocyte count of less than 1%, and only 2% nucleated red cells in the marrow with an essentially normal leukocyte formula. She has shown no response to therapeutic trials of cobalt, ACTH, riboflavin, and other conventional forms of therapy, such as liver, vit. B_{12}, etc. Iron turnover studies confirmed the other laboratory findings in that the marrow failed to accumulate and release radioiron for erythropoiesis. She has been diagnosed as erythroid hypoplasia. The urine concentrate

* This work was supported in part by the U. S. Atomic Energy Com.

† The diet (modified from McCollum's formula) consists of 67.5% whole wheat, 15% casein, 10% whole milk powder, 0.75% NaCl, 1.5% $CaCO_3$, 5.25% hydrogenated vegetable oil, and a concentrate of fish oil in amount to give 19 U.S.P. units of vit. A and 2.5 A.O.A.C. chick units of vit. D/g of diet.

‡ We wish to thank Dr. E. S. Evans for determinations of metabolic rate.

TABLE I. A Comparison of Hematologic Response of Normal Rats Injected with 1 ml of Untreated Urine Daily from a Normal and an Aplastic Anemia Donor.*

Material inj. 1 ml/day	Hct (%)	Red cell vol (ml/100 g body wt)	Total red cell vol (ml)	Increase in total red cell vol (%)
Saline control	45.6 ± 1.7†	2.39 ± .1	4.66 ± .2	
Normal urine	43.6 ± 2.6	2.32 ± .2	4.55 ± .2	0
Active "	49.9 ± 3.3	2.73 ± .2	5.34 ± .1	14.6

* 7 rats/group. † Mean and stand. dev.

was prepared by the ultrafiltration method described by Gorbman(15).

Results. A variety of normal human beings and patients with various hematological disorders were assayed for plasma erythropoietic activity by the Fe^{59} red cell incorporation assay using hypophysectomized rats. One ml of untreated plasma from normal human beings consistently gave an average Fe^{59} incorporation of 26%, as compared with 6% for the saline injected controls. No elevation in erythropoietic titer above normal was found in the patients assayed with the exception of 3 children with aplastic anemia, all of whom showed markedly elevated plasma erythropoietin, 1 ml of plasma producing a 40-50% incorporation of Fe^{59} in 16 hours. With such an elevation in plasma, it was decided to assay the urine. In 2 of the 3 children the erythropoietic activity of 1 ml of urine was as high as that of 1 ml of plasma, whereas normal urine shows no activity. In the third patient the urine showed no activity. If such an increase in iron incorporation after the administration of urine did in fact indicate a stimulation of erythropoiesis, then it should be possible to show an increase in the total red cell volume with a longer period of administration.

Table I compares the average values obtained in total circulating red cell volume with urine from the patient described above (1 ml daily for 14 days) with the values obtained with urine from a normal subject or with normal saline. The average hematocrit and red cell volume were significantly increased ($p = 0.001$, 15% above the saline-injected control) in the group injected with 1 ml of active urine. There was no change following injection of urine from a normal subject. Although the daily administration of only 1 ml of urine resulted in the production of a definite polycythemia in normal adult rats, it was felt that the true potency of this material could be demonstrated best by comparing the response obtained from larger amounts of urine with that obtained with the most effective known erythropoietic stimulant, hypoxia. For this purpose some method of concentration of the active principle from urine was sought. Since the ultrafiltration technic of Gorbman(15) provides a simple method for the concentration of gonadotrophic and possibly other pituitary hormones(16) in urine, the ultrafiltration of erythropoietically active urine was investigated. Under 20 lb nitrogen pressure, 500 ml of urine was forced through a collodion membrane. The membrane was dissolved in ether alcohol, and a precipitate was obtained by centrifugation. The precipitate was washed with ether, dried to a powder, and stored under vacuum. The powder was dissolved in saline, and an aliquot diluted to a volume equivalent to that of the starting material, while a second aliquot was dissolved so as to give a 13-fold concentration. The original urine, the ultrafiltrate, and the 2 dilutions of the residue remaining after ultrafiltration were assayed for erythropoietic activity by the Fe^{59} incorporation assay using normal rather than hypophysectomized rats. The use of normal rats in this assay has been

TABLE II. Partition of Erythropoietic Activity in Urine of a Patient with Aplastic Anemia.

Treatment	Vol equivalents inj. (ml)	Fe^{59} uptake* (16 hr) (%)
Starting material (untreated urine)	1	48 ± 6.3
Ultrafiltrate	1	28 ± 7.9
Residue after ultrafiltration	1	43 ± 7.6
Idem	13	61 ± 4.4
Uninjected control	—	23 ± 6.7

* Normal adult female rats; 10 rats/group.

TABLE III. Hematologic Values of Normal Rats Injected with Urinary Erythropoietic Fraction or Exposed to Simulated Altitude of 15,000 Ft for 14 Days.*

Treatment	Hct (%)	Red cell vol (ml/100 g body wt)	Total red cell vol (ml)	Increase in total red cell vol (%)
Saline	43.0 ± 1.66†	2.45 ± .1	4.48 ± .55	
Hypoxic control	49.9 ± 1.95	3.27 ± .2	5.76 ± .46	28.6
Urine fraction (2 mg/day)	56.8 ± 4.24	3.45 ± .3	6.70 ± .83	49.6

* 6 rats/group. † Mean and stand. dev.

found to be satisfactory when the activity of the material to be tested is sufficiently high. Table II summarizes the results of this assay. As can be seen from the table, no activity was found in the ultrafiltrate. The activity of the starting material and of the residue diluted to a volume equivalent to the starting material were equal. The more concentrated fraction showed a greater activity. All of the active material was recovered in the filter, providing a simple and efficient method for concentrating and purifying the activity and reducing it to a nontoxic, easily soluble powder. A urine fraction prepared in this way was injected subcutaneously into normal adult rats at a dose equivalent to 30 ml (2 mg) daily for 14 days. A second group was injected with saline and a third was maintained continuously in a decompression chamber at a simulated altitude of 15,000 feet for 14 days. On the 15th day total circulating red cell volumes were determined, and the animals were autopsied. Table III compares the average values for hematocrit and red cell volume for the 3 groups. As can be seen from the table, the total red cell volume of the rats maintained at a simulated altitude of 15,000 feet was 28.6% above that of the saline-injected controls, while those given an equivalent of 30 ml of active urine per day showed an increase in total red cell volume of 49.6% above the controls. Table IV compares average body weights, organ weights, and standard metabolic rates of the 3 groups. The rats injected with concentrate of active urine showed a greater increase in body weight than either the saline-injected or hypoxic controls, at least indicating that the injected material was not toxic. There was no significant change in the average weight of the endocrine organs or in the I^{131} uptake in the 3 groups. The spleens of the rats injected with urine concentrate showed a definite increase in weight, suggesting stimulation of erythropoiesis in that organ. The standard metabolic rate of the group injected with concentrate of active urine was only 9% above that of the saline-injected controls.

Thus, a certain group of patients classified as aplastic anemia produces and excretes erythropoietic substance in excessive quantities. It is suggested that in some cases the mechanism responsible for this high rate of production and excretion may be analogous to that responsible for the high level of follicle-stimulating hormone in the castrate or menopausal woman in that the production of the stimulant is uncontrolled because the target organ fails to respond, that is, there is no regulatory feedback mechanism. The source of production of the erythropoietic material found in plasma and urine has not been investigated, and one can only assume that that which is found in urine originates from that which is found in plasma.

TABLE IV. Organ and Body Weights and Metabolic Rates of Normal Adult Rats Injected with Urinary Erythropoietic Fraction or Exposed to Simulated Altitude of 15,000 Ft for 14 Days.*

Treatment	Body wt (g) Beginning	Body wt (g) Final	Adrenals (mg)	Thyroid (mg)	Ovaries (mg)	Uterus (mg)	Thymus (mg)	Spleen (mg)	Metabolic rate (cal/m²/hr)
Saline	171	183	59	12.5	67	388	245	845	31.5
Hypoxic control	171	177	51	13.1	65	304	192	890	
Urine fraction	171	194	59	13.0	67	281	211	1,305	34.4

* 6 rats/group.

Summary. 1) Some patients with aplastic anemia had exceptionally high levels of erythropoietic activity in plasma. The urine of these patients was assayed, and in some it was equally as potent as the plasma. As little as 1 ml daily for 14 days of untreated urine from such a patient produced a significant polycythemia in normal adult rats. When a concentrate of such urine, prepared by ultrafiltration, was injected at a dose equivalent to 30 ml daily for 14 days into normal adult rats, a polycythemia was produced which exceeded that resulting from exposure to a simulated altitude of 15,000 feet for the same period of time. 2) The high potency of such urine has allowed its assay in normal rats. This finding, together with the easy availability of urine and the efficiency of ultrafiltration as a method for preparing concentrates of urinary erythropoietic activity, should allow for a more rapid investigation of the chemistry and biology of this factor.

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Received September 10, 1957. P.S.E.B.M., 1957, v96.

Effect of Corticoids upon Skeletal and Renal Changes Produced by Stylomycin Aminonucleoside.* (23534)

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The literature on stylomycin aminonucleoside (SAN) is somewhat confusing, owing to the fact that the antibiotic now known as "stylomycin" was first called "achromycin"—a name which was subsequently transferred to another antibiotic(1)—and then "puromycin"(2,3,4). Little is known as yet about the pharmacology of this antibiotic, apart from its efficacy in the treatment of infections, particularly those caused by certain trypanosomes. It has been noted, however, that stylomycin and its aminonucleoside can produce a nephrosis-like syndrome with renal lesions, subcutaneous edema, pleural fluid, ascites, hyperproteinemia, hyperlipemia and azotemia in laboratory animals(1,3). This condition is accompanied by an increased aldosterone secretion(4), osteitis-fibrosa-like changes in the bones(5) and, in advanced intoxication, by an interstitial pancreatitis(6).

The object of the present communication is to report upon experiments which show that both the renal and the skeletal changes elicited by SAN overdosage depend largely upon the balance between pro- and anti-inflammatory corticoids.

* Supported by National Research Council of Canada and by Lederle Laboratories Division of Amer. Cyanamid Co., the latter having also supplied the stylomycin aminonucleoside used.