

## Erythropoietin in the Urine of Normal and Erythropoietically Abnormal Human Beings

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**A**LTHOUGH THE CONCENTRATION of erythropoietin may rise to very high and easily measured levels under abnormal circumstances, the concentration in normal animals of many species, including man, is not sufficient to be consistently demonstrated in unconcentrated samples of serum or urine.<sup>1-5</sup> The fact that the administration to normal animals of antibodies against erythropoietin results in cessation of erythropoiesis<sup>6</sup> is evidence that the hormone is essential for red cell production, and therefore must be present in low concentration in the body fluids of normal animals. Finne<sup>7</sup> recently reported that concentrates of 24-hour collections of urine from normal male human beings produced an increase in Fe<sup>59</sup> incorporation in polycythemic mice equivalent to that obtained with approximately 0.4 standard B units of erythropoietin. It has been estimated indirectly that the concentration in plasma of normal human beings is in the order of 0.003 standard B units per ml.<sup>8</sup>

To evaluate the relationship of erythropoietin to erythroid hypoplasia and mild degrees of hyperplasia, it would be advantageous to be able to measure the normal concentration. With presently available methods of assay, this requires concentrating the hormone from a large sample. As it may not always be possible to obtain large samples of plasma or serum, the urine was thought to provide the best starting material for detection of erythropoietin in normal human beings and patients with minimal acceleration or depression of erythropoiesis. This paper presents results using concentrates of human urine assayed for erythropoietin by the hypertransfused mouse Fe<sup>59</sup> uptake assay.<sup>9</sup>

### MATERIALS AND METHODS

Erythropoietin was concentrated from the urine by the collodion adsorption method.<sup>10</sup> All samples were assayed by the hypertransfused mouse Fe<sup>59</sup> red cell uptake assay of DeGowin et al.<sup>9</sup> Ten mice were used for each sample. In order to determine the amount of urine needed to extract measurable amounts of erythropoietin, the urine of a normal male subject was collected and processed over a 30-day period. The combined extract was

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assayed at different doses in order to establish the dose-response relationship. The erythropoietic activity was determined by comparison to a dose-response curve made using a laboratory standard of human urinary erythropoietin from a patient with aplastic anemia previously compared to standard A.<sup>o</sup>

Serum from rabbits immunized against human urinary erythropoietin was used to demonstrate that the response obtained with extracts of normal urine was due to erythropoietin. Serum containing antibodies against erythropoietin was prepared by the method of Schooley and Garcia.<sup>11†</sup> Erythropoietically active concentrate from the urine of a normal subject was incubated overnight in the cold with (1) 1.5 ml. of antibody-containing serum, or (2) 1.5 ml. of serum from normal rabbits, as a control. As a second control, 1.5 ml. of serum from normal rabbits was incubated with 0.9 per cent saline solution. This experiment was repeated using urine concentrate from another normal subject. The concentrate was incubated overnight in the cold with 0.1 ml. of serum from rabbits immunized against erythropoietin or with 0.1 ml. of serum from normal rabbits. The activity of the antibody-containing serum used in the second experiment was such that 0.1 ml. would neutralize 2 standard A units of erythropoietin.

From the dose-response curve obtained with extract of normal urine, it was determined that the extract of the entire urinary output for 72 hours would be required to obtain responses well above control values. For the remainder of the studies a 72-hour urine collection was used. Normal subjects and patients with hematologic disorders were provided with a deep-freeze, into which the urine was put immediately after voiding.

### RESULTS

The results obtained with increasing doses of extract from a single large collection of urine from a normal subject, and the results with increasing doses of a laboratory standard erythropoietin, are compared in Table 1. A definite dose-response relationship was established.

When the concentrates from normal human urine were incubated with rabbit serum containing antibodies to human urinary erythropoietin, all erythropoietic activity was lost, as judged by the hypertransfused mouse assay (Table 2). There was no loss of activity when the extract was incubated with serum from nonimmunized rabbits.

In order to determine the variation in amount of erythropoietin recovered from the urine, a 72-hour urine collection from 16 different normal subjects was compared with a second 72-hour urine collection made some time later of 5 of the same subjects (Table 3). As can be seen from the table, the amount of activity recovered from the urine of male subjects per day varied from 0.21 to 1.17 standard A units. The amount of activity recovered from the urine of female subjects varied from 0.16 to 0.32 standard A units per day. The average daily recovery of erythropoietin from the urine of the 10 male subjects was 0.54 standard A units; the average daily recovery from the urine of 6 female subjects was 0.22 standard A units.

Seven patients with well-documented primary polycythemia were studied. Six were classic examples of the disease with splenomegaly and elevated platelet and leucocyte counts. One was atypical in that there was no elevation of

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<sup>o</sup>Standard A was obtained from the Department of Biological Standards, Medical Research Council of Great Britain.

†We are indebted to Drs. Schooley and Garcia for preparing the antierythropoietin rabbit serum and performing the neutralizations.

**Table 1.—Erythropoietic Response of Hypertransfused Mice to Graded Doses of a Concentrate from Normal Human Urine or to a Laboratory Standard of Erythropoietin from Human Urine**

Dose	Fe <sup>59</sup> Uptake (%)
<i>Normal Human Urine Concentrate (mg.)</i>	
4	1.66 ± 0.55°
12	5.07 ± 0.97
36	16.81 ± 3.86
<i>Standard A Units of Erythropoietin†</i>	
.07	0.7
.09	5.4
.7	10.4
.9	14.3
9	25.4
Saline-Injected Control	0.2

°Standard error of the mean.

†From a male patient with aplastic anemia.

**Table 2.—Effect of Serum Containing Antibodies against Erythropoietin on Erythropoietic Activity of Normal Human Urine Concentrate**

Group	Fe <sup>59</sup> Uptake in Blood of Hypertransfused Mice (%)
Normal rabbit serum + 12 mg. of conc. from urine of a normal human being	5.22 ± 0.96°
Antibody + 12 mg. urine conc.	0.13 ± 0.02
Normal rabbit serum + saline	0.12 ± 0.02
Normal rabbit serum + 15 mg. of conc. from urine of a normal human being	6.71 ± 0.89
Antibody + 15 mg. urine conc.	0.15 ± 0.01
Saline-injected control	0.13 ± 0.01

°Standard error of the mean.

platelets or leucocytes and no splenomegaly; marrow distribution studies,<sup>12</sup> using the positron camera and Fe<sup>52</sup>, showed an isolated area of extremely active erythropoiesis in the left femur (Fig. 1). All had received treatment, either phlebotomy or P<sup>32</sup> or both. The response obtained with extracts of the urine of these patients was consistently below that obtained with extracts of urine from normal subjects residing at sea level, and 6 of the 7 showed no erythropoietic activity (Table 4).

Five healthy male residents at 17,000 feet (Chacaltaya, Bolivia) made 3-day collections of urine for this study.\* They had lived at 17,000 feet for 3–13 years, spending weekends at 11,800 feet (La Paz). Results from assay of urine concentrates are presented in Table 5. As can be seen from the table, there was a markedly greater recovery of erythropoietin, averaging 9.1 standard A units.

The erythropoietin content of a 72-hour urine collection from 5 patients with

\*The authors wish to thank Dr. Rafael Vidauwe and the staff of the Laboratorio de Físico Cosmico at Chacaltaya for their interest and enthusiastic cooperation.

**Table 3.—Average Daily Recovery of Erythropoietin from the Urine of Normal Human Beings**

	Vol. of Urine for 3 Days (ml.)	Mg. Injected per Mouse	Standard A Units/Day
Males	5700	16.5	0.59
	4300°	13.0	0.37
	4810	15.8	0.55
	5300°	15.0	0.31
	2600	17.2	0.37
	3620°	29.1	1.17
	2300	13.5	0.26
	1850°	9.6	0.21
	3600	14.2	0.55
	6600	14.5	0.50
	2650	15.2	0.46
	2800	20.6	0.45
	2210	27.2	0.70
	3480†	13.5	1.04
<i>Average</i>	3701		0.54
Females	2400	12.3	0.18
	3005°	13.7	0.16
	4800	8.4	0.24
	5985	24.5	0.18
	6925	10.9	0.32
	3200	16.1	0.16
	8990‡	17.3	0.29
	<i>Average</i>	5044	

°Collection and assay repeated.

†Child, age 13.

‡Kidney donor.

polycythemia secondary to renal pathology has been measured. All had elevated total circulating red cell volumes in the absence of evidence of cardiac or pulmonary disease. Two had simple renal cysts, two had malignant hypernephroma, and one developed evidence of increased red cell production beginning approximately 6 weeks after renal allograft for treatment of advanced, chronic, diffuse glomerulonephritis.<sup>9</sup> In only one, the kidney transplant, was more than a normal amount of erythropoietin recovered from the urine. Mirand et al. have recently reported increased erythropoietin in the plasma following renal transplantation in the rhesus monkey.<sup>15</sup> Recovery of erythropoietin from the urine of one patient with a polycythemia-producing cyst was below any measurable amount (Table 6). To rule out the possibility that the cyst-produced erythropoietin would not be recovered from the urine by collodion adsorption, 5 ml. of the cyst fluid were added to normal urine and the activity quantitatively recovered from the collodion.

°A complete report of this case has been published by Drs. Nies, Cohn and Schrier, Stanford University School of Medicine.<sup>13</sup> Positron camera photographs of the erythropoietic bone marrow distribution in this patient have been published (Fig. 2).<sup>14</sup>

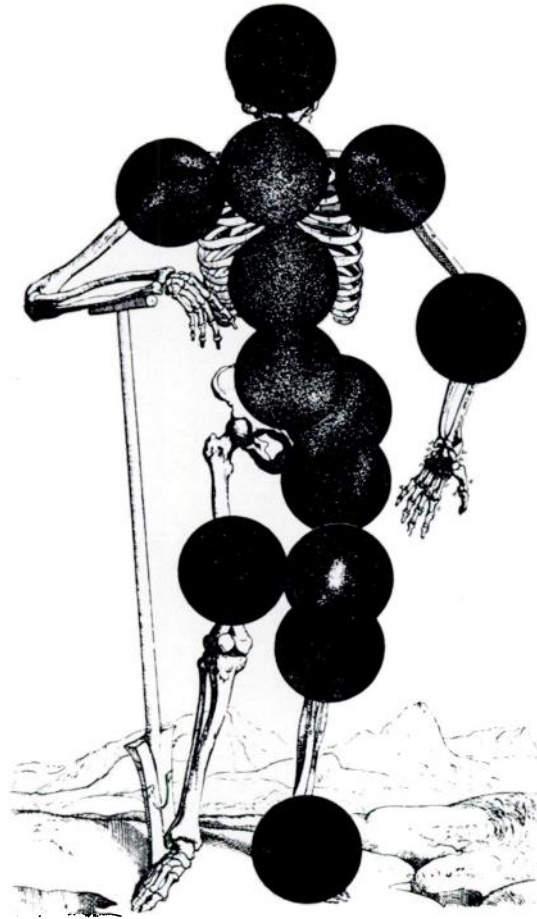


Fig. 1.—Composite of positron camera pictures of erythropoietic marrow distribution ( $\text{Fe}^{52}$ ) in a patient with atypical primary polycythemia. The distribution is normal except for the local area of intense activity in the lower portion of the left femur.

#### DISCUSSION

The necessity for concentrating the urine in order to obtain enough erythropoietin to assay has the disadvantage that one has no guarantee that the efficiency of extraction is the same for all samples. In patients in whom the erythropoietin concentration is high enough to be measured in an unmodified sample of urine, the efficiency of extraction by the collodion adsorption method has been estimated<sup>8,16</sup> to be from 28 to 100 per cent. Variation in extraction efficiency must be kept in mind when evaluating results obtained using concentrates.

It has been suggested that bacterial contamination may destroy erythropoietin.<sup>17</sup> None of the subjects studied had known urinary tract infection, but occult infection was not ruled out. The recovery of less erythropoietin from the

**Table 4.—Recovery of Erythropoietin from the Urine of Patients with Polycythemia Vera**

Hct. (%)	Hb (Gm./100 ml.)	Standard A Units/Day
52	14.2	0
48	12.0	0°
53	16.2	0
60	16.7	0
51†	15.3	0
60	16.7	0
47	14.5	0
54	15.0	0.18

°Collection and assay repeated.

†Female.

**Table 5.—Greater than Normal Recovery of Erythropoietin from Urine of Permanent Residents at 17,000 Feet°**

Hct. (%)	Hb (Gm./100 ml.)	Std. A Units Recovered per Day
50.3	16.0	1.14
54.7	18.2	4.0
51.0	18.0	8.6
55.5	18.5	9.5
61.5	19.8	22.4
<i>Average</i>		9.1

°Long-term (3–13 years) residents at Chacaltaya, Bolivia, with no known hematologic abnormalities beyond the elevated hematocrit and hemoglobin common to residents of their community.

urine of normal female subjects could be attributed to the greater bacterial contamination, but this was not studied.

It has been demonstrated (Table 1) that the greater the dose of extract of normal human urine, the greater the response. There are not sufficient data to determine whether the dose-response relationship is clearly the same or clearly different from that obtained with highly active human urinary erythropoietin. For the purposes of this study, all results have been compared to the more complete dose-response curve obtained with the laboratory standard expressed in standard A units.\*

The daily recovery of erythropoietin from the urine was approximately 0.5 standard A units for normal male subjects and 0.22 units for female subjects. The average daily urine volume was 1200 ml. for males and 1681 ml. for females, giving an average erythropoietin concentration per ml. of 0.0004 units for men and 0.0001 units for women. Assuming 50 per cent recovery in the process of concentration, the daily excretion would be 1 standard A unit for male subjects and 0.4 unit for female subjects, and the concentration in unmodified urine is estimated to be in the order of 0.0008 units per ml. for men

\*A comparison of the dose-response relationship of standard A (i.e., material previously related to standard A) to standard B has shown that these have the same activity in the assay system employed.

Table 6.—*Recovery of Erythropoietin from the Urine of Patients with Polycythemia Secondary to Renal Pathology\**

Diagnosis	Sex	Assay of Kidney Lesion	Plasma (Std. A Units per ml.)	Urine (Std. A Units per Day)
Simple cyst	M	Cyst fluid: 0.51 Std. A units per ml.	0	0
Hypernephroma	M	Acidified and boiled extract of tumor: 0.017 Std. A units per Gm.	0	0.6
Hypernephroma	F	Acidified and boiled extract of tumor: 0.46 Std. A units, total	—	0.3
Kidney transplant	F	—	—	2.7

\*A significant increase in total circulating red cell volume was demonstrated in all cases.

and 0.0002 units per ml. for women. This is approximately one-sixth the estimated concentration in normal plasma.<sup>8</sup> Hammond has estimated that at low levels of serum erythropoietin the difference between the serum level and the urinary level may be as much as 10- or 20-fold.<sup>18</sup> If this estimate is valid, then unmodified normal human plasma would contain 0.005–0.01 standard A units/ml. However, the recent finding of Mirand et al.<sup>5</sup> that erythropoietin can often be detected in unmodified normal human plasma (27 positive samples of 42) indicates that the normal plasma level is probably higher, near the minimum effective dose for the polycythemic mouse assay, 0.05 standard A units. Gordon et al.<sup>19</sup> state that in Cooley's anemia almost invariably the concentration of the erythrocyte-stimulating factor in plasma was greater than in urine, with the plasma-urine potency ratio usually ranging from 2 to 4.

The excretion of erythropoietin in the urine is of value only if it has some relationship to the concentration in body fluids or some constant relationship to abnormalities of red cell production. When the serum concentration is high enough to measure, there is always an increased urinary excretion of erythropoietin.<sup>18,20,21</sup> Concentrates of 72-hour urine samples from all patients studied with erythroid aplasia have shown normal or elevated erythropoietin content when unconcentrated samples of plasma or urine were negative. Patients with erythroid aplasia have been found to excrete from 1.28 to 25,000 standard A units of erythropoietin per day.<sup>8</sup> In this study of normal and low-level changes in urinary excretion, no correlation with serum concentration is possible. However, the recovery of 18 times as much erythropoietin from the urine of permanent residents at 17,000 feet is good evidence that changes in urinary excretion indicate changes in erythropoietin production. Other studies have demonstrated a positive correlation between erythropoietin excretion and blood loss anemia<sup>20</sup> or hemolytic anemia.<sup>19</sup>

In those patients with polycythemia secondary to renal cyst or carcinoma, and especially in those cases in which an increased concentration of erythropoietin can be demonstrated in the cyst fluid or in the tumor, there seems little doubt but that the concentration of erythropoietin in the blood is ele-

vated. The failure to recover erythropoietin from the urine of one such patient illustrates the need for caution in interpreting the meaning of the urinary erythropoietin level.

The recovery of erythropoietin from the urine of patients in the active phase of primary polycythemia was significantly less than that recovered from the urine of normal subjects and was usually below that which would be detected by the assay system employed. This was true in spite of current therapy with phlebotomy or P<sup>22</sup>. Nixon et al.<sup>22</sup> have suggested that the failure of patients with primary polycythemia to develop a significant concentration of erythropoietin in the serum following phlebotomy may be helpful in differentiating primary from secondary polycythemia.

#### SUMMARY

A standard method of concentrating urinary erythropoietin and a standard assay procedure can be used to demonstrate the hormone in the urine of normal human beings. The erythropoietically active material recovered produced increasing response to increasing dose and was completely neutralized by rabbit serum containing antibodies to human urinary erythropoietin. The average normal man excretes approximately 1 standard A unit of erythropoietin per day and the average normal woman excretes approximately 0.4 units per day. With the exception of one patient with a renal allograft, and normal subjects living at extremely high altitude, the recovery of erythropoietin from the urine has not been found to exceed normal in patients with polycythemia, whether the polycythemia is primary or secondary to renal pathology. Markedly elevated levels of erythropoietin were found in subjects living at extremely high altitude.

#### SUMMARIO IN INTERLINGUA

Un methodo standard pro concentrar erythropoietina urinari e un procedimento standard de essayage pote esser usate pro demonstrat ille hormon in le urina de humanos normal. Le assi obtenite erythropoieticamente active material produceva un accrescente responsa a un accrescente dose e esseva neutralisate completamente per sero de conilio a contento de anticorpore anti human erythropoietina urinari. Masculos normal exerce al media approximativemente 1 unitate standard A de erythropoietina per die, e femininas normal exerce al media approximativemente 0,4 unitates standard A per die. Con le exception de un patiente con un allografto renal, nulle patiente con polycythemia ha essite trovate con un excretion de erythropoietina urinari in excesso del norma sin riguardo a si le polycythemia esseva primari o secundari a pathologia renal. Marcatemente elevate nivellos de erythropoietina esseva trovate in subjectos residente a extreme altitudes.

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