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BIOLOGICAL AND CHEMICAL CHARACTERISTICS OF URINARY ERYTHROPOIETIN

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The remarkable constancy of red-cell number and red-cell volume in the normal organism indicates that a regulatory mechanism must exist that maintains the number of circulating erythrocytes at a constant level, stimulates erythropoiesis after hemorrhage or exposure to an atmosphere of reduced oxygen tension, and inhibits the production of red cells after hypertransfusion. That the mechanism set in motion by a change in oxygen tension is partially hormonal was indicated by injection of plasma from a bled rabbit into a normal rabbit by Carnot and Deflandre.[1] The intense recent interest in this subject was initiated by the parabiotic cross-transfusion experiments of Reissmann,[2] which demonstrated the presence of a humoral substance controlling erythropoiesis. In these parabiotic experiments, the bone marrow of a normal rat was stimulated through blood received from a hypoxic partner.

Since the work of Reissmann, there have been many reports of an erythropoietic stimulant found in plasma following administration of phenylhydrazine or after bleeding.[3,4] Hodgson and Toha[5] reported that an erythropoietic factor could be found in the urine as well as the plasma of rabbits made anemic by bleeding. Goldwasser et al.[6] reported erythropoietic activity in the plasma of rats after treatment with cobalt.

Reports from several laboratories have provided ample evidence that the level of erythropoietin in the circulation is considerably elevated in certain cases of anemia. Piliero et al.[7] and Medici et al.[8] have demonstrated erythropoietic activity in the filtrate of boiled plasma from several cases of Cooley's anemia, one of two cases of hypoplastic anemia, and one of two patients with sickle-cell anemia, as judged by stimulation of erythropoiesis in normal recipient rats. A similarly prepared extract of urine from one of Cooley's anemia subjects was also active. Gurney et al.[9] and Prentice and Mirand,[10] using the incorporation of Fe^{59} into the red blood cells of either hypophysectomized or starved rats, have demonstrated an increase in erythropoietic activity of plasma from patients with hypoplastic anemia, pernicious anemia, hemolytic anemia, leukemia, Hodgkin's disease, carcinoma of the cervix (receiving radiation), and following acute hemorrhage. In this laboratory it has been demonstrated that a concentrate of urine from certain anemic patients,

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injected daily for 14 days into normal adult rats, will produce a polycythemia that exceeds that resulting from exposure to a simulated altitude of 15,000 for the same period of time. These observations have led to the routine examination of both the plasma and urine of patients with a variety of hematological and other disorders.

MATERIAL AND METHODS

The erythropoietic activity of plasma and urine samples was assayed in either hypophysectomized or normal rats of the Long-Evans strain by the effect on Fe^{59} incorporation into red cells after 2 days of injection,[11] or the effect on total circulating red-cell volume as measured by the labeled cell-dilution method[12] after 14 days of injection. Samples of blood were collected in a heparinized syringe and centrifuged for 10 minutes, and the plasma was removed and frozen until injected. The plasma was not processed further. The urine samples were frozen immediately on collection and injected for assay either as native urine or as the residue from ultrafiltration prepared by the method of Gorbman.[13] All samples were injected subcutaneously, and female rats were used throughout this study. The hypophysectomized animals were operated at 28 days of age and used for the assay 8 days later. Normal rats weighing between 150 and 200 g were used for the remainder of the assays.

The 16-hour radioiron incorporation assay has been used in these studies as a screening test for erythropoietin, and an increase in the total red-cell volume in normal rats has been considered unequivocal evidence for erythropoietic stimulation.

RESULTS

Plasma from normal individuals and patients with various disorders was assayed, using the Fe^{59} red-cell-incorporation assay in hypophysectomized rats. The results are summarized in Table I. Plasma from four of the 22 patients increased the red-cell incorporation of radioiron above that seen following injection of normal plasma. Three of these 4 patients had a decreased rate of red-cell production (Cases 1, 3, and 4). Case 2 had a hemolytic syndrome, with an average red-cell life span of 30 days.

As has been reported previously, some patients with high plasma activity have high levels of erythropoietic activity in their urine.[14] Table II presents the Fe^{59} red-cell incorporation following injection of 1 ml of urine from normal individuals and patients with various hematological disorders as assayed in hypophysectomized rats. It can be seen that 1 ml of normal urine does not increase the incorporation of Fe^{59} into red cells, but that five of the 14 patients showed definite erythropoietic activity in the urine. Two of the patients showing high urinary activity had previously been found to have high plasma activity (Cases 1 and 2) and two had been found to have normal plasma activity (Cases 8 and 10). Also, two who had high plasma activity were found to have normal urinary activity (Cases 3 and 4).

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Table I

Effect of 1 ml of plasma from patients with various hematological disorders on radioiron incorporation into red cells of hypophysectomized rats

Case No.	Diagnosis	Fe^{59} uptake (% injected dose)	Interpretation
1	Erythroid hypoplasia	51.7 ± 6.1	High
2	Splenic syndrome	45.5 ± 12.5	High
3	Aplastic anemia	41.0 ± 7.3	High
4	Aplastic anemia	40.1 ± 8.6	High
5	Aplastic anemia	27.9 ± 7.6	Normal
6	Aplastic anemia	32.6 ± 6.8	Normal
7	Aplastic anemia	17.9 ± 5.3	Normal
8	Aplastic anemia	25.2 ± 10.8	Normal
9	Aplastic anemia	20.8 ± 7.9	Normal
10	Paroxysmal nocturnal hemoglobinuria	30.1 ± 10.6	Normal
11	Polycythemia vera	19.4 ± 8.8	Normal
12	Polycythemia vera	16.0	Normal
13	Polycythemia vera	26.0	Normal
14	Polycythemia vera	31.0	Normal
15	Polycythemia vera	31.0	Normal
16	Polycythemia vera	31.0	Normal
17	Polycythemia vera	28.0	Normal
18	Polycythemia vera	28.0	Normal
19	Hemolytic anemia	12.5 ± 5.6	Normal
20	Multiple myeloma	19.4 ± 9.0	Normal
21	Secondary polycythemia	21.7 ± 7.9	Normal
22	Chronic lymphatic leukemia	14.9 ± 8.4	Normal
	Normal	25.6 ± 7.1 (65 animals)	
	Uninjected control	7.4 ± 5.2 (414 animals)	

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Table II

Effect of 1 ml of urine from patients with various hematological disorders on radioiron incorporation into red cells of hypophysectomized rats

Case No.	Diagnosis	Fe^{59} uptake (% injected dose)	Interpretation
2	Splenic syndrome	48.9 ± 8.4	High
1	Erythroid hypoplasia	29.2 ± 7.0	High
10	Paroxysmal nocturnal hemoglobinuria	29.9 ± 6.4	High
23	Fanconi syndrome	19.9 ± 9.8	High
8	Aplastic anemia	19.1 ± 8.2	High
4	Aplastic anemia	8.8 ± 11.8	Normal
7	Aplastic anemia	8.7 ± 4.6	Normal
3	Aplastic anemia	10.8 ± 4.8	Normal
9	Aplastic anemia	9.9 ± 10.0	Normal
24	Polycythemia vera	7.9 ± 4.5	Normal
11	Polycythemia vera	5.6 ± 4.8	Normal
25	Secondary polycythemia	8.4 ± 6.7	Normal
21	Secondary polycythemia	11.5 ± 8.8	Normal
20	Multiple myeloma	6.6 ± 3.5	Normal
A	Normal donor	5.2 ± 2.9	
B	Normal donor	6.4 ± 9.0	
C	Normal donor	3.2 ± 5.4	
D	Uninjected control	7.4 ± 5.2	

Because the Fe^{59} red-cell-incorporation assay may give false positive results[15] and because of our own observation that 1 ml of boiled milk will give a definite response, it seemed essential to test the urines which showed high activity by some unequivocal method. We have previously reported that administration of active urine for 14 days to normal adult rats will produce a polycythemia,[14] and Medici et al. have reported that plasma from patients with Cooley's anemia was not only effective in increasing Fe^{59} incorporation, but prolonged administration to normal rats resulted in the production of a polycythemia.[8] In the present study, the urines that were found to have high activity by the Fe^{59} red-cell-incorporation assay were extracted by ultrafiltration and administered to normal adult female rats for 14 days. On the fifteenth day the total circulating red-cell volume was determined by the labeled red-cell-dilution method. Table III compares the results obtained with urine extract from four of the patients previously found to have high urine activity by the Fe^{59}-incorporation assay with the results obtained after exposure to a simulated altitude of 15,000 ft for 14 days and administration of saline. It can be seen from the table that urine extract from three of the four patients produced a polycythemia which exceeded that produced by a simulated altitude of 15,000 ft for the same period. Of the three patients who showed high urinary levels by the red-cell-volume assay, all had shown high activity in the urine and two (Cases 1 and 2) had shown high activity in the plasma by the Fe^{59}-incorporation assay. Urine from the fourth patient (Case 8), who had consistently shown high urinary activity and normal plasma activity by the Fe^{59} assay, was found to be essentially inactive when assayed by the red-cell-volume method.

Of the patients who were found to have elevated levels of erythropoietic activity by any of the criteria used in this study, two had hemolytic disease with low hemoglobin and very active marrow, and five were of the hypoplastic type, with a low hemoglobin and a hypoplastic marrow.

From the studies available it appears that a marked elevation of erythropoietin in the body fluids may be found in severe anemia but that a high level of erythropoietin does not consistently accompany severe anemia. There is nothing apparent in the clinical history of the individuals in which elevated levels have been found to indicate in which cases of anemia one might expect elevated levels of erythropoietin. Positive results have been obtained in approximately half the cases studied, those with hemolytic anemia being more consistently positive. Because not all severely anemic patients show an elevation of erythropoietin in plasma or urine, it must be admitted that we do not as yet understand the physiological or pathological mechanisms responsible. One must consider the possibility that some aplastic anemias exist because of a failure in production of erythropoietin.

With the demonstration that adequate amounts of highly potent erythropoietin can be obtained from the urine of some anemic patients, such material has been accumulated for biological and chemical investigations. The erythropoietin used in these studies was prepared by ultrafiltration from the urine of the following two patients:

Table III

Hematological response of normal rats to 14-day injection of urine concentrates from patients with various hematological disorders compared with response of normal rats exposed to simulated altitude of 15,000 ft for 14 days (7 rats per group)

Case No.	Diagnosis	Dose per day (mg)	Increase in total red-cell volume (% above control)
1	Erythroid hypoplasia	2.0	49.6
2	Splenic syndrome	1.3	37.0
10	Paroxysmal nocturnal hemoglobinuria	2.0	34.4
8	Aplastic anemia	2.0	7.5
Hypoxic animals			28.6
Saline			0

Case 1 is an 11-year-old Caucasian female first found to be anemic at the age of 1 1/2 months. She has received approximately monthly transfusions since then, and the hemoglobin has been maintained at 5 to 7g. When first examined in this laboratory five years ago, the skin color was found to be grayish-tan, the retinae and mucous membranes pale, and a hemic murmur was noted. The liver and spleen were not palpable at that time, but more recently some enlargement of the liver, and to a lesser degree the spleen, has been noted. The erythrocytes are normocytic and slightly hypochromic, and the reticulocyte count less than 1%. The leukocytes and platelets are within normal range. The nucleated red cells in the bone marrow are less than 2%, with a normal leukocyte distribution. She has shown no response to trials of cobalt, ACTH, riboflavin, and other conventional forms of therapy, such as liver and vitamin B_{12}. In a recent determination by the P^{32}-tagged-cell method, the red-cell volume was found to be 10.7 cc/kg of body weight (normal, 24 to 33). In vivo studies with Fe^{59} showed that the bone marrow failed to accumulate and release iron for erythropoiesis. The survival of the transfused erythrocytes appeared normal.

Case 10 is a 24-year-old Lebanonese student who had been in good health until 2 years ago, when symptoms of anemia first appeared and he reported pink urine. He was referred to this laboratory for study in May 1957, soon after his hemoglobin had reached a low of 3.5 g. The diagnosis of paroxysmal nocturnal hemoglobinuria had been made on the basis of a positive Ham test, the Crosby test, hemosiderin in the epithelial cells of the urine, persistent anemia, reticulocytosis, and marrow erythroid hyperplasia. On physical examination, his skin was observed to have a buff-yellow color, there was conjunctival pallor and slight left cardiac enlargement with a grade-I precordial systolic murmur. The liver and spleen were not palpable. He has a macrocytic anemia, and hemoglobin ranges from 5 to 7 g. Moderate hypochromia and some degree of anisocytosis and poikilocytosis has been noted. The reticulocytes range between 3 to 19%. Moderate leukocytopenia is present. Total serum bilirubin (Van den Bergh) was 1.868 mg% (direct 0.264 mg% and indirect 1.604 mg%); urine urobilinogen was 0.33 mg%/24 hr. Red-cell volume was 16.4 cc/kg of body weight (normal, 24 to 33). In vivo Fe^{59} studies revealed an iron-deficiency state and increased marrow erythropoiesis (about twice normal), with a secondary accumulation of iron in the liver and spleen. The mean erythrocyte life span was 27 days.

The material prepared from the urine of these two patients has consistently produced an increase in Fe^{59} incorporation and total red-cell volume in rats. In order to determine the range of species in which this urinary erythropoietin is effective, mice, rats, rabbits, dogs, and monkeys were given a dose of urinary erythropoietin ranging from 1.3 to 12.0 μg per gram body weight on two successive days. Twenty-four hours after the second injection, these animals and an equal number of controls were given a tracer dose of Fe^{59} intravenously. A sample of blood was removed 16 or 24 hrs later and counted for determination of the percent incorporation into circulating erythrocytes (Table IV). It was thought that the 16-hr interval which has been found satisfactory for rats might be too short in the case of the monkey. For this reason samples were collected at 24 hrs at which time the injected monkeys showed an increase, suggesting that within the variation of the method, the species responded similarly. In order to demonstrate conclusively the degree of erythropoietic stimulation represented by such a change in iron metabolism, groups of 10 mice, 6 rats, 2 rabbits, and 1

Table IV

Effectiveness of urinary erythropoietin in mammals judged by increase in Fe^{59} red-cell incorporation

No. of animals	Dose (mg)	Fe^{59} uptake[a] (%)	
		Control	Treated
Mice (10)	0.3	23.7 ± 7.2	54.5 ± 8.8
Rats (10)	2.8	23.0 ± 6.7	61.0 ± 4.4
Rabbits (2)	10.0	32.6 13.8	48.8 46.0
Dogs (2)	20.0	4.9 4.7	6.7 9.3
Monkeys (2)	20.0	2.4 17.5	9.2 23.0

[a]16-hr uptake except for monkey, which was 24-hr uptake.

monkey were given daily subcutaneous injections for 14 days. On the fifteenth day the total circulating red-cell volume was determined (Table V). The daily administration of this material for 14 days resulted in a 26% increase in the total circulating red-cell volume in mice, a 50% increase in rats, a 30% increase in rabbits, and an 11% increase in the monkey. Such responses in normal animals unequivocably demonstrate the erythropoietic potency of this material.

As a preliminary to any chemical characterization of this material, it was necessary to know the dose-response relationships by the use of the Fe^{59}-incorporation assay. For this purpose the effect on Fe^{59} incorporation of urinary erythropoietin in doses of 0.06 to 4.0 mg per day for 2 days was determined in groups of 10 normal rats. As can be seen from Fig. 1, the dose-response curve is relatively flat and one standard deviation is such that a several-gold difference in activity could not be accurately determined.

In order to recover the material collected on the collodion membrane after ultrafiltration, the membrane is dissolved in ether and alcohol, leaving the residue behind as an insoluble material. Thus, in the first step one finds that the erythropoietin is not soluble in ether or alcohol. Such an ultrafiltrate of urine is known to contain approximately 50% carbohydrate and 50% protein.[16]

The residue retains activity when placed in a boiling-water bath for up to 15 min (Table VI). If the active principle is a protein, this degree of resistance to boiling suggests an unusual class of protein.

The effect of trypsin digestion on the activity of the urinary erythropoietin was investigated. Seventy-five mg of residue from ultrafiltration was dissolved in 75 ml of saline and adjusted to pH 8. Twenty-five ml of this solution was frozen to be tested as the starting material 25 ml was incubated under toluene at room temperature for 8 hrs; and 25 ml was incubated with 3 mg crystalline trypsin under toluene at room temperature for 8 hrs. The results are summarized in Table VII. There was a definite loss of activity in the trypsin-treated portion, suggesting that erythropoietin contains peptide bonds within its structure. However, the loss of activity is not complete even after relatively long digestion and high enzyme-substrate ratio, which again points to some unusual class of protein.

A solution of active material from ultrafiltration was brought to half saturation and then full saturation by the addition of ammonium sulfate. The material was thus divided into 3 equal parts, two precipitates and the supernatant, as judged by optical density. Assay of these fractions by the Fe^{59}-incorporation method showed that the activity was equally distributed in the two precipitates and that no significant purification was achieved.

In zone electrophoresis on starch in a veronal buffer at pH 8.4, the activity migrated ahead of the position of the albumin-like component of urine. In experiments carried out with continuous-flow electrophoresis on a hanging curtain in 0.01 N acetic acid, evidence was also obtained for marked mobility toward the anode. These results indicate an isoelectric point at pH 4.5 or below.

Table V

Effect of injection of urinary erythropoietin in normal animals for 14 days

No. of animals	Daily dose (mg)	Increase in total red-cell volume (%)
Mice (10)	0.3	26.4
Rats (6)	2.0	49.6
Rabbits (2)	10.0	29.5
Monkey (1)	50.0	11.0

Table VI

Effect of boiling on the erythropoietic activity of the residue from ultrafiltration (tested in normal rats)

Treatment	Fe^{59} uptake (%)
Starting material	47.6 ± 10.3
Boiled 5 minutes	44.9 ± 8.7
Boiled 15 minutes	41.6 ± 7.9
Uninjected control	26.0 ± 6.7

Table VII

Trypsin digestion of urinary erythropoietin as measured by Fe^{59}-incorporation in red cells (10 rats per group)

Treatment	Fe^{59} uptake (%)
Starting material	45.6 ± 11.8
Incubated at room temperature for 8 hrs	45.1 ± 7.8
Incubated with trypsin at room temperature for 8 hrs	36.0 ± 15.6
Saline-injected control	21.7 ± 10.2

When the residue is dissolved at a concentration of 20 mg per ml and the pH of the solution adjusted to 3.0 with 0.1 N HCl, a precipitate, amounting to 20 to 30% of the material, is formed. This precipitate contains the activity, as measured by the red-cell-volume assay.

The most recent work on the chemical characteristics of the plasma erythropoietin is that of Rambach, et al.,[17] who find evidence that the erythropoietic activity of plasma is in the form of a mucoprotein traveling with an alpha-2 globulin. This plasma erythropoietin resists boiling and is extractable with 5% perchloric acid, which is a characteristic of mucoproteins.[18] The fact that the urinary erythropoietin is neither precipitated nor inactivated on boiling, is not completely destroyed by prolonged digestion with trypsin, is precipitated at pH 3, and is present in the residue remaining after ultrafiltration which contains a large amount of carbohydrate suggests that the urinary material may be a mucoprotein.

On the basis of these observations a series of highly purified mucoprotein fractions from normal human plasma obtained from Schmid,[19,20] Winzler and associates,[21] and Deutsch,[22] were assayed for erythropoietic activity by the Fe^{59}-incorporation method (Table VIII). As can be seen from the table, the only fraction that had any suggestion of activity was the Zn a_2 fraction from Schmid. Because these fractions represent the range of mucoproteins in normal plasma, it is a question whether erythropoietin is not represented here or whether normal plasma does not contain measurable amounts of erythropoietin.

Table VIII

Comparison of erythropoietic activity of highly purified mucoprotein fractions from normal human plasma and urine concentrate from a case of hemolytic anemia, by the use of the Fe^{59}-incorporation assay

Material	No. of animals	Total dose (mg)	Fe^{59} uptake (%)
Zn α_2	10	15	34.5 ± 11.3
Σ-VI barium precipitate	10	50	32.1 ± 11.3
α_1 Acid glycoprotein	10	40	31.3 ± 14.8
Orosomucoid	10	25	30.1 ± 13.0
Fetuin	10	25	23.4 ± 9.6
Urine concentrate	10	3	51.1 ± 6.6
Uninjected controls	20	-	28.1 ± 10.8

SUMMARY AND CONCLUSIONS

Of 25 patients with various hematologic and other disorders who were studied by the Fe^{59}-incorporation assay, two showed high levels of erythropoietic activity both in plasma and urine, two showed high levels in the plasma but no activity above normal in the urine, and 1 showed high activity in the urine but no increase in activity above normal in the plasma. The significance of the elevated urinary activity was further demonstrated by injecting extracts of the urine from those patients who showed high erythropoietic activity by the Fe^{59} assay into normal rats for 14 days. The urine of three of the four patients tested in this way produced a polycythemia that exceeded that produced by exposure of normal rats to a simulated altitude of 15,000 ft for the same period of time. It is suggested that our knowledge of the mechanisms responsible for an elevated erythropoietin in the body fluids may be advanced more quickly by the simultaneous assay of both urine and plasma of patients with various hematological disorders and by evaluating the significance of the Fe^{59} red-cell-incorporation assay by determining whether those samples that appear to be active are capable of producing a polycythemia when injected into normal rats.

Erythropoietically active material prepared from the urine of one case of aplastic anemia and one case of paroxysmal nocturnal hemoglobinuria was effective in producing a polycythemia in normal rats, mice, rabbits, and one monkey when given daily for 14 days.

The fact that the urinary erythropoietin is neither precipitated nor inactivated on boiling is not completely destroyed by prolonged digestion with trypsin, is precipitated at pH 3, and is present in that part of the residue remaining after ultrafiltration that contains a large amount of carbohydrate suggests that the urinary erythropoietin may be a mucoprotein. Similar evidence, along with the demonstration that plasma-erythropoietin activity is soluble in 5% perchloric acid, has suggested that the plasma erythropoietin is also a mucoprotein. Evidence is presented, however, that indicates some differences in chemical properties of plasma and urinary erythropoietins.

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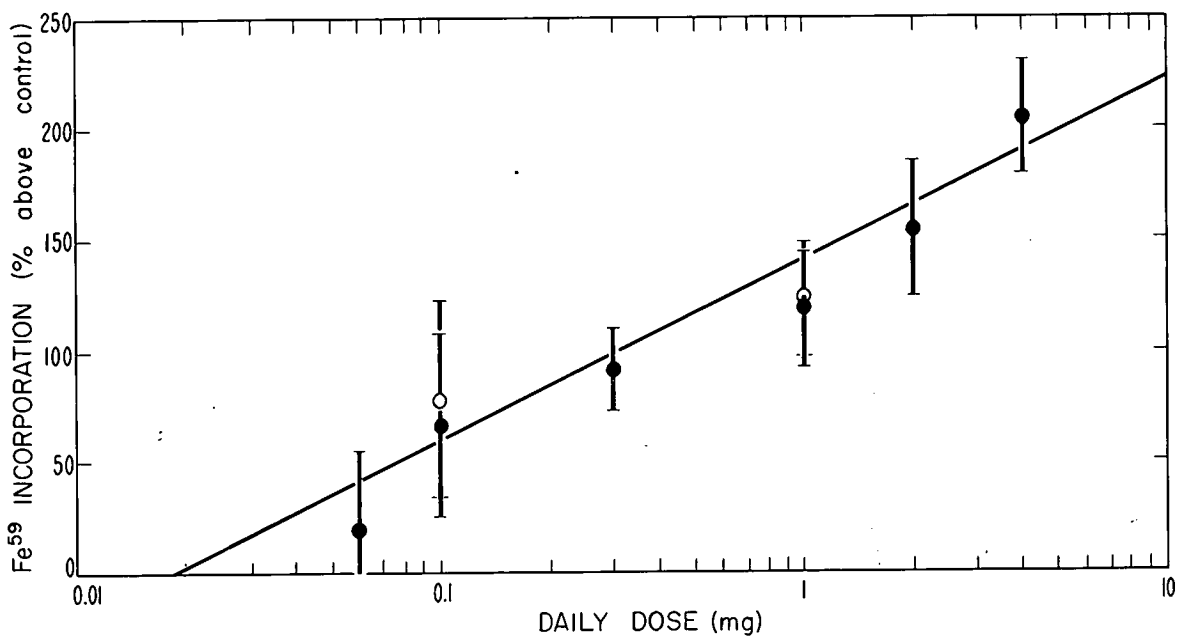

Fig. 1. Dose-response curve for urinary erythropoietin by the Fe^{59} red-cell incorporation assay using normal adult rats.

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