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Assay of "Hemopoietine" in Starved Animals; Properties of Urinary Hemopoietine. (24274)

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The effects of plasma from anemic rabbits on plasma iron turnover of normal rabbits have been described(1). A clear increase in plasma iron turnover was produced by native and boiled plasma from phenylhydrazine treated rabbits; bled rabbits' plasma produced a slighter effect and only after dialysis. Although rich in Hemopoietine, plasma from phenylhydrazine treated animals is not an economic source for abundant material necessary for chemical fractionation studies. In view therefore of our previous findings that "Hemopoietine" appears in urine(2,3), it was decided to use urine from anemic animals, as a source of "Hemopoietine" for chemical studies. Before doing this it was necessary, however, to work out a satisfactory method for Hemopoietine concentration, and also have available an adequate test for assaying the various fractions quantitatively. Use of plasma iron turnover studies in normal rabbits as a routine assay is impractical, because of large amounts of material required. Preliminary experiments with normal rats, measuring Fe^{59} uptake in erythrocytes, indicated that this was not a satisfactory bio-assay animal either. It was with great interest therefore that we read of the experiments which demonstrated that animals with depressed erythropoiesis, presumably a consequence of low endogenous hemopoietine titers, were very sensitive to extracts from plasma of anemic animals(4,5).

This communication presents results of experiments in which starved rats with depressed erythropoiesis(5), were used as assay animals to develop a method for Hemopoietine concentration, and to describe the application of this method to the study of some properties of urinary Hemopoietine.

Method donor animals. Rabbits of both sexes, average weight 2 kg, were used. Treatment of these animals has been described(1). For urine collection the rabbits were maintained in metabolic cages, and urine collected in sterile glass flasks kept in an ice-salt mixture. The urine obtained was centrifuged and then kept frozen until further treatment.

A. *Receptor rabbits.* To test the effect of Hemopoietine on Hb synthesis in starved animals, extract of boiled plasma from phenylhydrazine treated rabbits prepared according to Borsook(6), were injected intravenously (10 ml/kg), twice a day for 2 days, in fasted male rabbits (2 kg). Animals were fasted 2 days before and during injection period. Fe^{59} was injected on morning of 5th day of fasting, and plasma iron turnover and Fe^{59} incorporation into red cells studied by methods previously described(1). The results of this experiment are summarized in Table I.

B. *Receptor albino rats.* In preliminary tests for adequate method of concentration for Hemopoietine, starved male albino rats, of weights indicated in Table II, were used. These rats were fasted 2 days previous to Hemopoietine injections and for 3 days thereafter. Hemopoietine preparations were injected intravenously (tail vein) twice a day on third and fourth day of fasting. Fe^{59}, 0.2

TABLE I. Plasma Iron Turnover and Fe^{59} in Erythrocytes of: Normal Rabbits (N.R.), Starved Rabbits Injected with: Saline (S.R.S.), Normal Rabbits' Boiled Plasma (S.R.N.R.P.) and Phenylhydrazine Treated Rabbits' Boiled Plasma (S.R.Ph.R.P.).

Group	No. rabbits	Plasma iron turnover time constant, %/hr	Plasma iron, μg/ml	Plasma iron turnover, μg/hr	Erythrocytes Fe^{59}, % dose 24 hr	48 hr
N.R.	5	43.2 ± 1.6	2.93 ± .31	106 ± 5.2	49.7 ± 5.0	60.9 ± 4.0
S.R.S.	5	22 ± 3.15	2.76 ± .83	51.2 ± 4.5	12.8 ± 1.6	36.9 ± 6.9
S.R.N.R.P.	6	18 ± 3.85	3.48 ± .42	56.7 ± 6.2	15.1 ± 3.1	38.1 ± 8.1
S.R.Ph.R.P.	9	56.4 ± 1.23	1.97 ± .31	97.4 ± 8.4	48.1 ± 4.6	74 ± 4.1

μc/rat was injected on morning of 5th day of fasting, approximately 18 hr after last Hemopoietine injection. Fe^{59} was injected intravenously (femoral vein) under light ether anesthesia, as iron-ascorbate, incubated in normal rat serum. An aliquot of dose diluted to 10 ml was kept as standard. 20 hr after Fe^{59} injection, the animals were anesthetized with ether and exsanguinated by aortic puncture using a heparinized syringe. Blood radioactivity was determined in 2 ml sample using a scintillation counter. Hematocrits were done using standard technics. In a small group of starved rats, total circulating red cell volume was determined after 5 days of starving using Fe^{59} tagged red cell method. The average value obtained: 2.29 ± 0.13 ml/100 g (pre-fasting weight) was used for calculation of Fe^{59} uptake in erythrocytes.

C. *Starved grey rats.* The studies described above were continued using male grey rats obtained from stock of Instituto de Biología Juan Noe, University of Chile. It was necessary to vary the technic a little. The receptor rats were fasted for 4 days, received subcutaneous injections of Hemopoietine, twice a day on 2nd or 3rd day and again in morning of 4th day of fasting, 1 hour prior to Fe^{59} injection. Doses given are shown in Table III 24 hr after Fe^{59} injection, the animals were anesthetized with ether, exsanguinated by aortic puncture, and liver removed for radioactivity measurements. In preliminary experiments, measuring femurs and spleen as well, most valuable information could be obtained from counting only liver and blood. In some of these starved rats total red cell volume was determined by the Fe^{59} tagged red cell method, on morning of 5th day of fasting. Advantage was taken of this experiment to determine amount of blood radioactivity remaining in liver after exsanguination. The mean circulating red cell volume in these animals was 2.42 ± 0.14 ml/100 g (pre-fasting weight) and 2.13% of total blood radioactivity was present in the liver corresponding to 100 g rat (pre-fasting weight). These values were used for calculation of Fe^{59} in erythrocytes and liver.

Results. A. Effect of boiled plasma from phenylhydrazine treated rabbits on plasma iron turnover and Fe^{59} incorporation into red cells in fasted rabbits. The results summarized in Table I show that fasting lowers plasma iron turnover to about 50% of its normal value. Fe^{59} incorporation into eryth-

TABLE II. Fe^{59} in Erythrocytes of Starved Male Albino Rats. Rats of groups 1 to 3 weighed from 180-220 g; those of groups 4-9 between 250-320 g. Abbreviations: B.P., extract of boiled plasma; R., rabbit; N., normal; Ph., phenylhydrazine treated; Al. ext., alcoholic extract; Bl., bled; U., urine.

Group	No. animals	% Fe^{59} erythrocytes	Dose of material inj./rat
(1) Saline	8	4.7 ± 1.37	8 ml 9‰ NaCl sol.
(2) B.P.N.R.	6	7.4 ± .97	8 " extract from 6 ml boiled plasma
(3) B.P.Ph.R.	15	37.7 ± 3.10	*Idem*
(4) Saline	11	11.7 ± 2.05	8 ml 9‰ NaCl sol.
(5) $B.P.Ph.R._{I}$	5	42.4 ± 4.20	10 mg Biuret positive material
(6) Al. ext. $B.P.Ph.R._{I}$	7	43.7 ± 3.42	*Idem*
(7) Al. ext. $B.P.Ph.R._{II}$	6	32.0 ± 3.60	"
(8) Al. ext. $U.Ph.R._{II}$	6	43.6 ± 4.20	"
(9) Al. ext. U.Bl.R.	4	24.3 ± 5.85	"

TABLE III. Fe^{59} (% of Injected Dose) in Erythrocytes and Liver of Starved Rats, Average Weight 160 g, Injected with Crude Alcoholic Extract of: Normal Rabbits' Urine (C.N.R.U.), Phenylhydrazine Treated Rabbits' Urine (C.Ph.R.U.) and Alcoholic (Al.) Extracts of the Latter.

Group	No. animals	Erythrocyte Fe^{59}, %	Liver Fe^{59}, %	Erythrocyte liver ratio, E/L	Relative specific activity, %
(1) Saline	13	10 ± .79	17 ± 1.22	.59 ± .07	
(2) C.N.R.U. 10 mg	6	11.5 ± 2.48	16.7 ± 1.91	.75 ± .22	
(3) C.Ph.R.U. 10 mg	6	50.7 ± 1.83	7.2 ± 1.25	8.0 ± 1.23	100
(4) 50% Al., pH 7, Ph.R.U. 10 mg	6	32.5 ± 2.6	10.3 ± .93	3.27 ± .46	36
(5) 50% Al., pH 4, Ph.R.U. 4 mg	6	30.7 ± 1.89	8.5 ± .75	3.7 ± .39	105
(6) C.Ph.R.U. 16 mg	4	41.8 ± 2.82	5.8 ± .44	7.4 ± .96	100
(7) 50% Al., pH 4.5, C.Ph.R.U. 16 mg	6	19.7 ± 3.5	9.9 ± 1.3	2.2 ± .18	24
(8) 50%-80% Al., pH 4.5, C.Ph.R.U. 16 mg	6	60.2 ± 5.14	4.9 ± .18	13.7 ± 2.48	193*

* Relative specific activities are calculated as ratio $\frac{\triangle \text{E/L/dose x}}{\triangle \text{E/L/dose crude}}$, where $\triangle$E/L is increase of E/L ratio; dose x, dose of purified material, and dose crude, starting material.

rocytes, especially at 24 hr, is also notably diminished. Extracts of boiled plasma from phenylhydrazine treated rabbits increase plasma iron turnover and Fe^{59} uptake in erythrocytes significantly. Extracts of boiled normal rabbit plasma do not affect these indexes.

B. *Effect of various "Hemopoietine" preparations on Fe^{59} uptake by red cells in starved albino rats.* Table II summarizes results on effect of Hemopoietine on Fe^{59} uptake by erythrocytes of starved male albino rats. Extracts of boiled plasma from phenylhydrazine treated rabbits, notably increases the percentage of Fe^{59} appearing in red cells at 20 hr. (II-3). Extract of boiled normal plasma produces a slight increase of Fe^{59} incorporation, when compared with control and experimental series run simultaneously (II-1-2). The factor responsible for the effect of extracts of boiled anemic plasma is non-dialyzable. It flocculates on treatment with saturated $(NH_4)_2SO_4$ at pH 4.5. On treatment of water extracts obtained from boiled plasma of phenylhydrazine treated rabbits with 4 vol. ethanol at pH 4.5 an abundant precipitate formed, which was easily separated from supernatant by centrifuging. The precipitate obtained was extracted with $9^0/_{00}$ NaCl solution, and the soluble fraction was tested for activity. Table II (5,6) shows results of assay of this type of crude alcoholic fraction of boiled plasma, which has the same activity as original boiled plasma extract. The dose was 10 mg Biuret reacting material/rat. All the Biuret reacting material in solution precipitated on treatment with phosphotungstic acid, not as in the case of the aqueous extract of boiled plasma, in which only a part of the Biuret reacting material came down with phosphotungstic acid. In view of the positive result of this assay, it was decided to test for activity in extracts obtained in a similar fashion from urine collected from rabbits after 3 bleedings (once a day, 15 ml/kg) and urine from phenylhydrazine treated rabbits, collected after 5 injections of phenylhydrazine (10 mg/kg). The phenylhydrazine treated donors were exsanguinated after urine collection and the alcohol extract of boiled plasma also tested for activity. Table II (7,8,9) shows clearly that the activity of extracts of urine from phenylhydrazine treated donors, is greater than that of boiled plasma of the same donors, and the latter is greater than that of the urine of bled rabbits.

The crude urinary extract was tested for presence of phenylhydrazine using a Fiegel spot test, sensitive to 0.04 μg(7) and shown to give no color reaction.

C. *Bio-assay and properties of urinary Hemopoietine.* Fig. 1 shows the results of a quantitative bio-assay, using crude alcoholic extract of urine of phenylhydrazine treated rabbits. In Fig. 1a it can be seen that Fe^{59} of erythrocytes at 24 hr is proportional

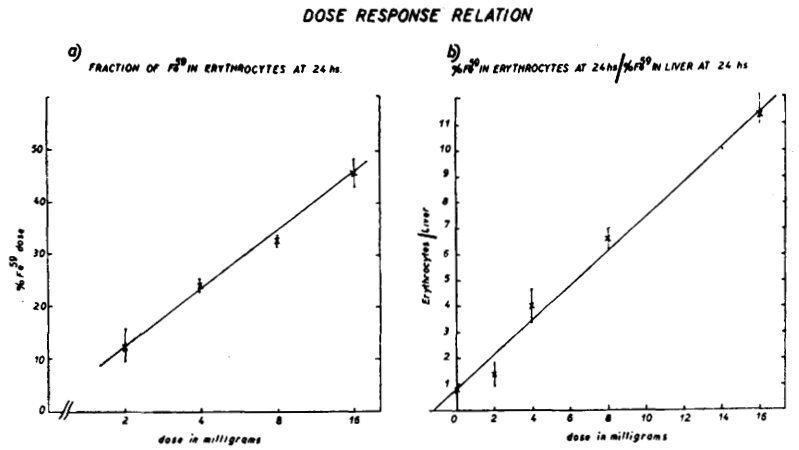

FIG. 1. Starved male rats inj. with different doses of Biuret reacting material of urine of phenylhydrazine treated rabbits.

($r > .994$) to $\log_2$ of dose of urinary Hemopoietine, expressed as mg Biuret reacting material. Fig. 1b) shows that the ratio Fe^{59} of erythrocytes/Fe^{59} of liver at 24 hr is proportional to dose of urinary Hemopoietine ($r > .998$). Table III shows results of other experiments with Hemopoietine in grey rats. The most active urinary fraction to date was obtained from the crude alcoholic precipitate of anemic urine, by the following procedure: the precipitate was extracted with 0.1 m acetate buffer pH 4.5 and the solution treated with one vol. ethanol. The precipitate which formed showed lower specific activity than the crude extract (Table III-6-7); the supernatant however, on treatment with another 3 vol. of alcohol gave a precipitate whose specific activity is greater than that of the crude extract (Table III-8). Table III-2 shows that the extract of normal rabbit's urine has no effect. The precipitate which forms on treating the crude extract at pH 7 with 1 vol, alcohol has low activity, while that which forms on lowering pH to 4 has as high specific activity as the starting material (Table III-3-4-5).

Discussion. The observations on starved rabbits clearly show that there is a notable decrease in Hb synthesis as reflected by low plasma iron turnover and low incorporation of Fe^{59} in erythrocytes. The analysis of Fe^{59} uptake in red cells at 24 and 48 hr suggests that aside from a decrease in iron consumption by bone marrow, there is also in this case an increase of maturation time of red cell precursors in bone marrow, which makes the estimate of erythropoiesis, as measured by uptake at 24 hr alone, lower than it actually is. The plasma iron turnover in fasted rabbits is as low as the minimum value observed by Bothwell *et al.*(8) in experiments in which X irradiation was used to suppress red cell formation. But in the case of starvation, the depression of erythropoiesis is not a consequence of marrow damage, but probably reflects low Hemopoietine titers(5) and can be reversed by administration of exogenous Hemopoietine, in the form of extract of boiled plasma from phenylhydrazine treated rabbits. The effect of this plasma extract is probably specific, and not the consequence of administration of amino acids, glucose and a few milligrams of protein that are present in the extract, as extract of boiled normal plasma, which conceivably contains similar quantities of these components, has no significant effect. The quantities of Hemopoietine in normal plasma were probably in this case insufficient to obtain effective blood concentration.

The observations on starved rats confirm the finding of depressed erythropoiesis(5) in these animals and show that they are practical assay animals for Hemopoietine. Simultaneous study of Fe^{59} in red cells and liver shows that low erythrocyte Fe^{59} in starved rats is not only a consequence of delayed maturation

of bone marrow precursors, but reflects a change in iron distribution with smaller uptake by the erythroid marrow and a greater proportion of iron going to body stores, as represented by the liver. Hemopoietine injection increases iron uptake by marrow and diminishes that of the stores. The significant linear correlation between Erythrocyte Fe^{59} and $\log_2$ dose, and Erythrocyte/liver Fe^{59} ratio and dose of Hemopoietine, demonstrates the feasibility of carrying out quantitative bio-assays, using the criterion of specific activity in chemical fractionation studies of Hemopoietine. In comparing Fig. 1-a and 1-b, it can be seen that the measurement of E/1 ratio permits detection of smaller amounts of Hemopoietine than does the measurement of Fe^{59} in erythrocytes alone; 2 mg and 4 mg of crude urine alcoholic extract respectively.

If one analyzes that data obtained in normal rats taking into account the dose response relationship shown in Fig. 1, some interesting conclusions can be drawn with respect to assay of Hemopoietine in normal animals. Thus Fe^{59} uptake in normal rats (32%) is seen to correspond to uptake of starved rats injected with 8 mg urinary extract. The increase of Fe^{59} uptake observed by us in normal animals, on receiving 10 mg of an extract of boiled plasma (32.8 to 44.9%) is in the range of what would have been predicted by the dose-response curve of Fig. 1. Thus a dose of 10 mg given to a starved rat increases Fe^{59} uptake 3 fold, while a similar dose given to normal animal increases uptake in only 37%. The gain in sensitivity obtained by using starved animals is manifest.

The presence of "Hemopoietine" activity in urine of bled and phenylhydrazine treated animals, confirms the results of previous experiments(2,3) and accords with the findings of Piliero *et al.*(9), Van Dyke *et al.*(10) and Winkert *et al.*(11) in urine from anemic patients. The fact that extracts of urine from phenylhydrazine treated rabbits are more active than those from bled rabbits is in agreement with the findings that plasma from phenylhydrazine treated animals has more erythropoietic activity than that of bled donors(1,12). The abundance of Hemopoietine in phenylhydrazine treated animals' urine presents a valuable source of material for study, as it is not difficult to maintain rabbits for prolonged periods of time in a state of chronic hemolytic anemia, and obtain several liters of urine from them.

The evidence so far presented by various laboratories points to the protein(13) or polypeptide nature of Hemopoietine, and suggests that it may be classified as a mucoprotein (14,15). The properties here described for Hemopoietine obtained from urine and plasma accord with the properties described for mucoproteins, as chemical analysis of active fractions shows that they contain more than 10 mg total neutral sugar (Orcinol) per 100 mg protein (Biuret).

Summary. 1. Fasted rabbits and rats were used as assay animals for Hemopoietine preparations. Depressed erythropoiesis manifests itself by low plasma iron turnover and uptake of Fe^{59} in erythrocytes in rabbits, and by low erythrocyte and high liver Fe^{59} in rats. 2. Injections of extracts of boiled plasma from phenylhydrazine-treated rabbits increases plasma iron turnover and Fe^{59} uptake by erythrocytes in rabbits, and produces an increase in erythrocyte Fe^{59}, accompanied by a drop of liver Fe^{59} in rats. Uptake of Fe^{59} in erythrocytes is proportional to log dose of Hemopoietine administered, while Fe^{59} erythrocyte/liver ratio is proportional to dose. Active fractions have been prepared from both urine and plasma of anemic rabbits by alcohol fractionation. The active material is non-dialyzable and contains more than 10 mg carbohydrate per 100 mg protein.

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Production of Diabetes Insipidus in the Rat.* (24275)

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Reports dealing with diabetes insipidus in partially hypophysectomized rats have seldom described operative technics(1-6). This report sets forth in detail the surgical technic of neurohypophysectomy as performed in over 500 animals during the past 5 years, and also more recent experience with hypothalamic lesions produced with a stereotaxic instrument. The water exchange in acute and chronic DI as well as other conditions resulting from such lesions will also be discussed.

Methods. Sprague-Dawley rats of either sex, weighing approximately 200 g, were subjected to neurohypophysectomy by the parapharyngeal approach. Clean but not sterile technic was used. The procedure as described by Smith(7) and Thompson(8) has been modified in 2 important respects: 1) ether is used as the anesthetic because it avoids respiratory depression often seen with barbiturates, and 2) after making a midline neck incision extending from lower jaw to sternal notch, the trachea is incised just below the thyroid cartilage parallel and between 2 rings of tracheal cartilage. A glass tube 4 mm in diameter, 5-6 cm long and tapered to 2 mm at the end serves as a tracheal cannula thru which ether may be administered. Except during actual administration of the anesthetic, it is important to remove ether bottle to avoid CO_2 accumulation and hypoxia. A bloodless plane of dissection between deep strap muscles on the right side of neck can be found by gently retracting with 2 pairs of curved forceps. The digastric muscle is not cut as by Thompson(8) because retraction of the trachea permits adequate exposure of the basisphenoid area of skull. Two curved paper clip retractors are now placed in the plane separating the deep neck muscles dissected on the right side. The exposed basisphenoid area, which can be recognized by the V-shaped muscles inserting along the midline, is freed of its muscle attachment. An important landmark can now be seen—intersection of a longitudinal ridge (crista occipitalis) with the blue transverse cartilage (occipito-sphenoid synchondrosis). Approximately 1 mm rostral to this intersection a hole is drilled and enlarged with a No. 9 dental burr (a smaller size burr may be used to start the hole). Two precautions should be observed during drilling; occasionally a blood sinus in the bone located in rostral portion of hole may be entered, resulting in a brisk oozing of blood which can be stopped only by sustained pressure best applied using #3 round dental pel-

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