Effect of Plasma from Bled and Phenylhydrazine Treated Animals on Plasm Iron Turnover. A Test for "Hemopoietine". (23621)

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Experiments carried out in several laboratories point to the existence of a factor capable of stimulating erythropoiesis, "Hemopoietine" of Carnot and Deflandre(1), in plasma of animals in which red cell formation has been increased by bleeding(2), phenylhydrazine(3), and exposure to low barometric pressures(4). In working with this factor it is necessary to have an adequate, rapid biologic test for assaying its activity. The majority of workers give several injections of plasma, or plasma extracts from anemic animals, usually phenylhydrazine treated rabbits, to normal rats, and observe the increase in one or all of the following: Hb concentration, hematocrit, red cell and reticulocyte counts. Some workers obtained increase in Hb concentration(3,5) others only in red cell count with no increase in Hb and hematocrit(6). The reticulocyte responses observed in some experiments are quite striking, in others not at all clear cut. These tests are time consuming, use indirect methods for measuring erythropoiesis and raise the question of validity of utilizing another species, the rat, as an assay for activity of an erythropoietic factor, possibly a mucoprotein(7) obtained from the rabbit. Recent studies with Fe^{59}(8) have shown that the plasma iron turnover is a good index of Hb synthesis. It was therefore decided to investigate the effect of plasma and plasma extracts of anemic rabbits, on plasma iron turnover in normal rabbits, with a view to developing a useful biologic test for study of the properties of the erythropoietic factor (or factors) "Hemopoietine," present in the blood of animals with increased erythropoiesis.

Material and methods. Rabbits of both sexes, average weight 2100 g (1600-2400), were used as experimental animals. The rabbits, kept in individual cages and given a standard diet obtained from the San Cristobal Milling Co., Santiago, Chile, were divided into groups of donor and receptor animals. As donors, normal rabbits, bled rabbits and rabbits treated with phenylhydrazine were used. The receptors were normal rabbits. The plasma from bled rabbits was obtained on the 4th or 5th day after daily bleedings of 15-20 ml/kg when Hb levels were about 5 g%. The phenylhydrazine treated animals were exsanguinated after 6 days of treatment with 7.5 mg/kg daily of this drug. Average Hb concentration at the time of bleeding was 4 g%. Heparin was used throughout the experiments as anticoagulant. In some experiments plasma was injected in the native state; in others plasma dialyzed 24 hr against 30 volumes of .9% NaCl solution, or plasma extracts prepared according to Borsook(3) were used. In one experiment an acid acetone extract of bled rabbit's plasma was injected. In all experiments plasma, or plasma extracts were injected intraperitoneally in the receptor rabbits in doses of 10 ml/kg twice a day for 2 days. Plasma iron turnover studies were made approximately 16 hr after the last plasma injection. Two types of control experiments were run, normal untreated rabbits and rabbits injected with normal rabbit's plasma. Rabbits with over 11.5 g% Hb and less than 3% reticulocytes, in good physical condition, were used as normals. For determination of plasma iron turnover $Fe^{59}Cl_3$ of high specific activity (1-3 c/g) obtained from Oak Ridge, was used. Previous to injection the $Fe^{59}Cl_3$ was transformed into Fe^{59} ascorbate(9) and incubated for 10′ at room temperature with serum, the unsaturated iron binding capacity of which was measured previously(9). Dose of Fe^{59} injected intravenously was approximately 1 μc/rabbit; an aliquot of the injected Fe^{59} serum was diluted to 50 ml, with .9% NaCl solution and kept as a standard. Blood samples (3 ml) were obtained from animals 20′, 40′, 60′ and 80′, 24 and 48 hrs after Fe^{59} injection. Radioactivity

TABLE I. Mean Values for Plasma Iron in 10 Rabbits at 20′, 40′, 60′ and 80′ after Injection of Fe^{59}.

Time	20′	40′	60′	80′
Plasma iron, μg/ml	2.17	2.11	2.18	2.20

TABLE II. Fe^{59} Incorporation into Red Cells, 24 and 48 Hr after Injection in Normal Rabbits, in Which No Samples for Plasma Radioactivity Measurements Were Taken, and in Normal Rabbits in Which Sampling Was Done at 20′, 40′, 60′ and 80′ after Fe^{59} Injection.

Group	No. of rabbits	Fe^{59} incorporation into red cell (% inj. dose) 24 hr	48 hr
NR, no samples	5	16.4	27.0
NR samples	7	35.7	64.4

of plasma samples was measured with NaI (Tl) Scintillation Counter (Philips, Eindhoven); 3000 counts were totalized in each sample. The cpm/ml plasma *vs.* time, were plotted on semi-log paper and a line fitted to the points. The plasma iron turnover time-constant K, was calculated from the following equation: $K = \frac{0.693}{T½}$, where T½ is the plasma radioactivity half life determined graphically. Correlation analysis carried out in 25 individual experiments showed that a significant linear correlation existed between log cpm/ml plasma and time ($-r > 0.98$). In view of this, calculation of turnover time constants was carried out as indicated previously. Volume of distribution of Fe^{59}-transferrin, was calculated from the following equation: $Vd = \frac{D}{cpm/ml_{to}}$, where Vd = volume of distribution, D = dose of Fe^{59} injected, expressed as cpm and cpm/ml_{to} = cpm/ml at zero time, as obtained from graphic extrapolation, of the line relating log cpm to time. Plasma iron concentration was determined by the method of Ramsay(10). In preliminary experiments, plasma iron determinations were carried out in each of the samples drawn at 20′, 40′, 60′ and 80′. It was seen from these experiments that the fluctuation of plasma iron levels during the period of sampling was not marked (Table II). In view of this, iron determinations for plasma iron turnover calculations were made using pooled plasma from the 4 blood samples. With reference to accuracy of the method, duplicate determinations of plasma iron showed a maximum variation of .2 μg/ml and a series of 10 determinations of iron, in a standard solution of iron containing 2 μg/ml, gave a coefficient of variation of 2.9%. Plasma iron turnover was calculated from total plasma iron, multiplied by K (the turnover constant). Fe^{59} incorporation into red cells was also measured in these experiments. For calculation of percentage of Fe^{59} appearing in the circulating red cells, the blood volume of the rabbit was taken to be 57 ml/kg. This value was obtained by us in measurements carried out in 20 normal rabbits, using Evans Blue and P^{32} labelled red cells. Percentages of Fe^{59} incorporation into red cells are included for purposes of illustration, but do not have quantitative value, since we have observed that the blood sampling of 3 ml at 20′, 40′, 60′, and 80′ for plasma radioactivity measurements, markedly influences the incorporation of Fe^{59} into red cells (Table I). However, the Fe^{59} uptake measurements are useful in that they show the trend in the different experiments in which plasma samples were taken.

Results. The results are summarized in Table III in which the following abbreviations have been used. NR = normal rabbits. NRP = normal rabbits' plasma. BRP = bled rabbits' plasma. BRDP = bled rabbits' dialyzed plasma. Acet BRP = acetone extract of bled rabbits' plasma. PhRP = phenylhydrazine treated rabbits' plasma. PhRBP = phenylhydrazine treated rabbits' boiled plasma. Table III shows values for plasma iron turnover and red cell incorporation of Fe^{59} in the above mentioned groups. Plasma from phenylhydrazine treated rabbits markedly increases plasma iron turnover, while plasma from bled rabbits, although producing a significant increase in the percent per hour turnover, does not increase plasma iron turnover, since it produces a marked drop in plasma iron: 208 to 126 μg bled rabbits' plasma after dialysis does however increase plasma iron turnover and its effect on plasma iron is less marked. Normal rabbits' plasma

TABLE III. Plasma Iron Turnover and Fe^{59} Incorporation into Red Cells in Normal Rabbits (NR) Injected with: Normal Rabbits' Plasma (NRP), Bled Rabbits' Plasma (BRP), Bled Rabbits' Dialyzed Plasma (BRDP), Acetone Extract of Bled Rabbits' Plasma (Acet BRP), Phenylhydrazine Treated Rabbits' Plasma (PhRP) and Boiled Plasma of Phenylhydrazine Treated Rabbits (PhRBP). Values indicated are means and standard errors. Mean values for plasma iron turnover are not calculated from the product of the mean values of plasma iron and the iron turnover time constant, but are means of the individually calculated plasma iron turnover.

Group	No. of rabbits	Plasma iron turnover time constant (%/hr^{-1})	Total plasma iron (μg)	Plasma iron turnover (μg/hr^{-1})	Fe^{59} incorporated into red cells (% doses) 24 hr	48 hr
NR	8	58.0 ± 4.54	208.0 ± 18.8	116.0 ± 13.9	35.7 ± 3.14	64.4 ± 3.1
NRP	10	61.1 ± 4.4	182.0 ± 13.3	108.0 ± 9.25	54.0 ± 2.92	80.5 ± 1.85
BRP	13	99.5 ± 3.27	126.0 ± 9.0	124.6 ± 8.77	53.6 ± 1.85	91.6 ± 1.41
BRDP	9	114.0 ± 6.8	144.0 ± 10.86	157.0 ± 8.26		95.2 ± 1.92
Acet BRP	4	59.5 ± 4.07	90.5 ± 6.52	53.5 ± 4.55		73.5 ± 4.22
PhRP	15	117.0 ± 6.07	224.2 ± 15.63	260.0 ± 17.86	72.3 ± 3.5	(72 hr) 74.7 ± 5.11
PhRBP	9	159.0 ± 9.4	163.0 ± 23.7	272.0 ± 37.12	68.4 ± 3.5	(72 hr) 84.4 ± 4.61

does not affect plasma iron turnover. With reference to the values for Fe^{59} incorporation into red cells the trend is for all plasma injections to produce an increase in Fe^{59} uptake. PhRP produces a maximum uptake at 24 hr, while NRP and BRP show maximum uptake at 48 hr, with the BRP group showing larger uptake at 48 hr than the NRP group. The supernatant obtained after boiling plasma from PhRP at pH 5.5 produces a marked increase in plasma iron turnover in spite of the fact that the plasma iron is slightly lowered. On the other hand, the acid acetone extract of bled rabbits' plasma notably lowers plasma iron turnover and plasma iron.

Discussion. Plasma from phenylhydrazine treated rabbits produces a clear cut increase in plasma iron turnover and in the rate of incorporation of Fe^{59} into red cells, when injected into normal rabbits. These probably reflect an increased Hb synthesis in these animals. Plasma from bled animals does not increase plasma iron turnover for although it markedly increases the speed of Fe^{59} disappearance, it also lowers plasma iron. The finding emphasizes the importance of measuring plasma iron in this type of study. The experiments with dialyzed bled rabbits' plasma and with acetone extracts of this plasma point to the existence in the blood of severely bled rabbits of at least 2 factors influencing iron metabolism: one nondialyzable which increases iron turnover and another dialyzable, soluble in acid acetone, which lowers plasma iron concentration and plasma iron turnover.

The finding that boiling does not inactivate PhRP disagrees with experiments of Erslev (11) but accords with the results of Borsook *et al.*(3) Gordon, *et al.*(5) and Linman and Bethell(6). However it is important to point out that the conclusions of the latter, who suggest that the factor is probably nonprotein, are open to doubt, as we have observed that the supernatant from boiled plasma treated with perchloric acid, contains a significant amount of phosphotungstic acid precipitable material which reacts with Biuret and has a carbohydrate content of around 4 mg/100 mg protein (seromucoid).

The percentage of the injected Fe^{59} appearing in the red cell at 24 and 48 hr is a function of many variables: size of plasma labile iron pools, uptake of iron by the erythroblasts and rate of release of red cells containing labelled hemoglobin from the marrow. Thus the index of Fe^{59} uptake when used alone, does not necessarily indicate the state of erythropoiesis. This point is illustrated by the results of our experiment with Acet BRP and PhRP, in which the 48 hr Fe^{59} uptakes are similar and yet plasma iron turnover is 5 times smaller in one group than in the other.

Conclusions. 1) Plasma obtained from phe-

nylhydrazine treated rabbits markedly increases plasma iron turnover. The supernatant obtained after boiling this plasma retains its effect on iron turnover. 2) Bled rabbits' plasma does not affect iron turnover, but has a clear cut lowering effect on plasma iron. Dialyzed plasma of bled rabbits, however, increases plasma iron turnover, while acetone extracts of bled rabbits' plasma, significantly depress plasma iron and plasma iron turnover. 3) The fraction of Fe^{59} appearing in the red cells at 24 and 48 hr is increased by normal plasma, bled rabbits' plasma, phenylhydrazine treated rabbits' plasma and is not a good index of the state of erythropoiesis. 4) Analysis of the effect of anemic rabbits' plasma on iron metabolism as studied with tracer doses of Fe^{59} gives information on the factors controlling iron metabolism and presents a practical method for bioassay of the plasma erythropoietic factor "Hemopoietine."

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Further Studies on Diuresis in Normal and Adrenalectomized Rats Following Total-Body X-Irradiation.* (23622)

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Experiments on changes in water balance in relation to total-body irradiation have been carried out by France(1) and Edelmann(2,3) and in relation to partial-body irradiation as well by Smith and Tyree(4). Both polydipsia(4) and polyuria(5), which occur in rats following total-body exposure, have been shown to be dependent upon the presence of the adrenal cortex. Removal of the anterior pituitary prior to irradiation eliminates polyuria, but removal of the posterior pituitary (5) does not. Certain irregularities appear in the literature concerning changes in water balance following irradiation. For example, Smith and Tyree(4) found that only a certain percentage of rats manifested polydipsia following irradiation. This percentage could be increased by increasing the x-radiation dosage. Also, Kay and Entenman(6) report that irradiation of rats 1, 3, and 7 days following adrenalectomy does not prevent diuresis.† In view of these and other inconsistencies, it was believed that the influence of additional factors in relation to water balance and irradiation should be examined.

Factors that might be relevant were the state of hydration of the animals at the time of exposure, water intake following irradiation, body weight, and length of time that elapsed between adrenalectomy and x-irradiation. Olewine and Perlmutt(7) were able to show that while irregularities in potassium ex-

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