put. In animals with abnormal hemolysis, on the other hand, input from destroyed cells is high and initially above output; plasma iron thus rises to a certain level when, mainly as a consequence of increased red cell production, output again equals input.

Summary. 1) The relation between plasma iron turnover and plasma iron concentration is studied in normal and fasted rabbits, in bled and phenylhydrazine treated rabbits, and in rabbits treated with extracts of plasma of bled and phenylhydrazine-treated donors. 2) In normal, phenylhydrazine-treated and starved rabbits, plasma iron turnover is independent of iron concentration over the range of 2-5 μg Fe/ml; below this range plasma iron turnover in normal rabbits tends to drop. Bled rabbits and rabbits receiving extracts with erythropoietic activity show lower plasma iron concentrations than normal, and in these animals plasma iron turnover depends on iron concentration.

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Effects of Urinary Hemopoietine on Fe^{59} Distribution in Rats Studied While Plasma Fe^{59} is High. (25868)

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In the majority of studies in which Fe^{59} has been used in rats to assay extracts with erythropoietic activity, the criterion employed has been the fraction of injected Fe^{59} (% Fe^{59}) appearing in erythrocytes from 16-24 hours after tracer injection(1,2,3). Although extracts rich in erythropoietic activity increase erythrocyte Fe^{59} uptake, this method does not permit quantification of increase in erythropoiesis. Determination of plasma iron turnover and uptake of iron by marrow would give data more directly related to magnitude of erythropoiesis(4,5). Since, when large numbers of rats are employed, it is not practical to carry out accurate plasma iron turnover measurements requiring serial blood samples, we devised a technic in which plasma iron turnover and mean plasma Fe^{59} specific activity (between 0 - t hours) can be estimated from one plasma sample taken at a time (t hours), when plasma Fe^{59} is still high in controls. From mean plasma specific activity and % uptake by different organs, quantity of iron going to these organs can be calculated. We here describe the technic and its application to assay of urinary erythropoietic activity. The results of this method are compared with 24 hour Fe^{59} assay.

Methods. Extracts with erythropoietic activity were obtained from urine of phenylhydrazinize-treated rabbits and patients with aplastic anemia by methods described elsewhere(6). The kaolin adsorption procedure used was similar to that employed by Winkert *et al.*(7). Grey rats, both sexes of a x c strain obtained from Inst. de Biol. Juan Noé,

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Univ. de Chile, were used either in normal condition or fasted 4 days. Weights are given in the Tables. Injections of extracts were made on 2nd and 3rd day of fasting, and Fe^{59} was injected(3) on morning of 4th day. Some animals were bled at 3 hours after tracer injection when starved animals were used, and at 1½ hours when recipients were normal. Preliminary experiments showed that at these times about 40% of radioiron was still in the plasma. The animals, anesthetized with ether, were bled from the aorta, using a heparinized syringe. Plasma was separated by centrifugation, and red cells were washed thrice with cold 0.9% NaCl solution Radioactivity of red cells, liver and both femurs was measured in well type scintillation counter, and activity was compared with standards of comparable volume. Plasma iron concentration was measured by method of Schade (8). On morning of 5th day of fasting, 24 hours after tracer injection, the rest of the animals were bled and radioactivity in washed red cells was measured. For calculation of percentage injected Fe^{59} in plasma, plasma volume was taken as 3 ml/100 g weight. For calculation of total red cell Fe^{59} uptake, red cell volume was taken as 2.3 ml/100 g weight. Values were obtained from experiments in which red cell volume was measured using erythrocytes tagged with Fe^{59}. Radioactivity in organs was corrected for that contributed by residual blood by factors obtained in animals killed 3 and 6 minutes after injection of serum and red cells tagged with Fe^{59} respectively.

To calculate plasma iron turnover and plasma mean specific activity, it was assumed that in rats(9,10), as in rabbits(5) and humans(4), plasma Fe^{59} radioactivity declines as a simple exponential function of time. Thus: Plasma $Fe^{59} = 1.e^{-Kt}$, where K = plasma iron turnover rate(9), or fraction of plasma iron turned over per unit time. Given plasma Fe^{59} at time t, it is possible to obtain the value of K using tables of exponential function or from $K = 1/3 \ln \frac{1}{F_t}$ (F_t = fraction of injected Fe^{59} remaining in plasma at time t). With the value of K, plasma iron turnover (P.I.T.) $\mu g/$ hr is calculated as: P.I.T. = K x Fe x P_v (Fe = plasma iron concentration, P_v = plasma volume). With these data, mean plasma specific activity $\overline{SA}_p$, between 0-t hours, can be calculated from $\overline{SA}_p = \frac{1}{Fe \times P_v \times t \int_0^t e^{Kt} dt} = \frac{1 - F_t}{P.I.T. \times t}$. From $\overline{SA}_p$ an estimate can be made of amount of iron which flows to any organ from the plasma compartment. Thus iron flux to the liver Q_L equals: $Q_L = \frac{\%\ Fe^{59}L}{\overline{SA}_p \times t}$ (% $Fe^{59}L$ = % injected Fe^{59} in liver at time t). This calculation is based on the assumption that loss of radioiron from liver between 0-3 hrs is negligible. In the case of marrow it is not possible to make this assumption because appreciable amounts of Fe^{59} appear in red cells at 1½ and 3 hours in animals treated with erythropoietically active extracts. In these experiments, the flux of Fe to the marrow Q_m is taken as approximately equal to total amount of iron leaving plasma minus that going to non Hb-forming tissues. The latter was assumed to equal twice the liver iron flux because preliminary studies of Fe^{99} distribution in viscera of the rat, made 3 hrs after tracer injection, demonstrated that liver contained as much radioiron as the sum of that found in heart, lungs, gastro-intestinal tract and kidneys, after appropriate corrections were made for radioactivity contributed by remaining blood. Therefore, Q_m = P.I.T. − 2 Q_L. The validity of this calculation is supported by the finding that in normal rats the fraction of injected Fe^{59} in erythrocytes at 48 hours closely approximates the ratio of Q_m/P.I.T.

Results. (Table I.) In fasted rats injected with urinary hemopoietine plasma radioactivity is much lower than in control rats injected with saline or with extracts of urine from normal rabbits. Estimated values for P.I.T. and Q_m are higher and are a function of amount of extract injected. Erythrocyte Fe^{59} is higher in hemopoietine-treated rats than in saline controls. Animals receiving extracts of urine from normal rabbits have higher erythrocyte Fe^{59} than saline controls and yet have similar P.I.T. and Q_L. Injection of hemopoietine

TABLE I. Fe^{59} Distribution at 3 Hours in Starved Female Rats, and at 1½ Hr Post Tracer Injection in Non-fasted Rats. Values indicated are means and standard errors. Plasma iron concentration (Fe) was measured in samples of pooled plasma. Duplicate measurements agreed within 3%.

	Group	No.	Plasma Fe^{59}	Erythrocyte Fe^{59}	Liver Fe^{59}	Femur, Fe^{59}	E.P.I.T.	E.Q.L.	E.Q.M.	Plasma (Fe)
			% dose				μg/hr			μg/ml
(a)	Fasted ♀ receptors (140 g)	22	27.6 ± 1.0	1.60 ± .17	15.3 ± 1.1		6.8	1.45	3.9	3.8
	N.R.U., 6 mg	11	21.0 ± 3.1	4.0 ± .48	18.8 ± .6		6.4	1.29	3.8	2.5
	Hp, 6 mg	8	5.6 ± .50	3.9 ± .58	7.4 ± .42		10.7	.83	9.0	2.7
	,, 12 ,,	6	3.8 ± .38	7.0 ± .30	6.2 ± .51		15.0	.97	13.0	3.3
(b)	Fasted ♀ receptors (180 g)	5	41.2 ± 1.45	.53 ± .11	11.5 ± 6	2.7 ± .34	4.8	.9	3.0	3.1
	3 hr post tracer inj. $CoCl_2$, 10 μM	4	32.1 ± 2.13	3.3 ± .95	13.9 ± .72	4.9 ± .43	6.4	1.3	3.8	3.3
	,, 20 ,,	5	16.4 ± 2.67	6.2 ± 1.46	13.0 ± .47	6.0 ± .38	7.3	1.2	4.9	2.3
	H.U.Hp	6	3.7 ± 23	30.2 ± 1.12	4.2 ± .1	5.4 ± .2	9.4	.4	8.6	1.7
(c)	Non-fasted ♀ receptors (180 g)	7	38.1 ± 1.2	4.5 ± .3	7.3 ± .46	3.6 ± .19	7.8	1.9	4.0	2.3
	1½ hr post tracer inj. $CoCl_2$, 10 μM	6	40.2 ± 1.51	3.5 ± .37	8.9 ± .26	3.2 ± .11	10.1	3.0	4.1	3.3
	,, 20 ,,	6	37.3 ± 1.3	4.4 ± .69	8.6 ± .14	3.3 ± .13	10.8	3.0	4.7	3.3
	Hp (18 mg)	7	22.5 ± 1.13	15.2 ± 1.49	9.2 ± .58	4.4 ± .36	16.8	3.9	9.0	3.3
	Kaolin									

E.P.I.T. = Estimated plasma iron turnover. E.Q.L. = Estimated flow of iron to liver. E.Q.M. = Estimated flow of iron to marrow. N.R.U. = Material precipitating with 4 vol ethanol at pH 4.5 from normal rabbit urine. Hp. = Material precipitating at pH 4.5 with 4 vol ethanol from urine of phenylhydrazinize-treated rabbits. H.U.Hp. = Human urinary hemopoietine; material precipitating with 4 vol ethanol at pH 4.5 from urine of patient with aplastic anemia, dose/rat = material obtained from 30 ml urine. HP Kaolin = Hemopoietine obtained from urine of phenylhydrazine-treated rabbits by adsorption on kaolin, elution with 1 M NH_4OH and precipitation with 5 vol acetone at pH 4.5.

in starved rats increases Femoral Fe^{59} but lowers Liver Fe^{59}. The response of normal rats to hemopoietine (Table Ic) is similar to that of starved rats except for Liver Fe^{59} and Q_L which increases. Table I also shows the effects of 10 and 20 μM $CoCl_2$, a substance known to stimulate erythropoiesis, on Fe^{59} distribution in fasted and normal rats. Like hemopoietine, cobalt decreases plasma Fe^{59} and increases erythrocyte Fe^{59}. However, cobalt differs from hemopoietine in that it increases, rather than lowers Liver Fe^{59} and Q_L in fasted rats. In non-fasted rats the doses of cobalt used do not affect plasma, Erythrocyte Fe^{59} or Femoral Fe^{59}. They do however increase Liver Fe^{59}. Table Ib shows that fasted rats injected with urinary hemopoietine or 20 μM cobalt, which show the most marked changes in Fe^{59} distribution, also have lower plasma iron concentration.

Fe^{59} in red cells is an exponential function of the fraction of plasma iron turned over per hour (%/hr) (Fig. 1a). A 2-fold increase of plasma iron turnover rate is accompanied by an 8-fold increase in Erythrocyte Fe^{59}, while for a 3-fold increase in turnover rate, Erythrocyte Fe^{59} is about 30 times the average control values.

Fig. 1b summarizes results of experiments in which Fe^{59} distribution was studied in 16 groups of fasted male rats (at least 4 animals per group) at 3 hours and in another 16 groups at 24 hours after tracer injection.

FIG. 1a. Relation between erythrocyte Fe^{59} 3 hr post tracer inj. (ordinate), and fraction of plasma iron turned over/hr (K %/hr) (abscissa) in: ■ Fasted controls rats; × fasted rats inj. with preparations of urinary hemopoietine.

FIG. 1b. Relation between % Fe^{59} in erythrocytes at 24 hr (ordinate) and that present at 3 hr (abscissa).

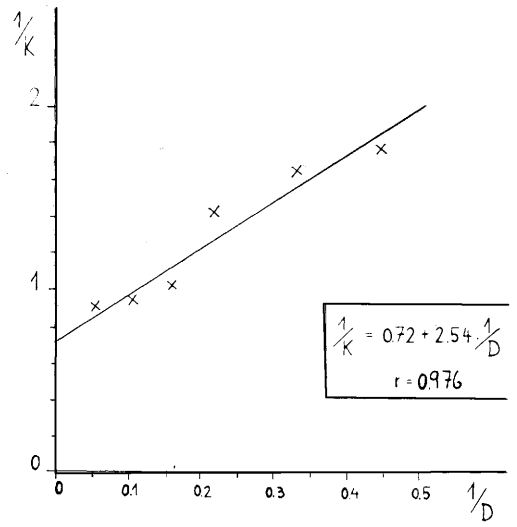

FIG. 2. Relation between reciprocal of K, fraction of plasma iron turned over/hr (ordinate), and reciprocal of dose (D) of rabbit urinary hemopoietine inj. (abscissa).

Seven of the groups were controls, and the other 11 received preparations of urinary hemopoietine from phenyl-hydrazine treated rabbits or patients with aplastic anemia. A significant linear correlation exists between Erythrocyte Fe^{59} at 24 hours and that at 3 hours ($r = 0.985$).

Fig. 2 shows the relation between reciprocal of dose (D) of rabbit urinary hemopoietine and reciprocal of effect as measured by K%/hr, fraction of plasma iron turned over per hour. This type of dose effect analysis was proposed by Stetten(11). The results show a significant linear relationship ($r = 0.976$) between 1/K and 1/D. From the parameters of this line, one may calculate the maximum effect, and dose of rabbit urinary hemopoietine producing ½ maximal effect: 139%/hr and 3.52 mg respectively.

Discussion. The increase in P.I.T. in hemopoietine treated rats probably results from increased flow of iron to bone marrow, evidenced by increased Fe^{59} uptake in femurs and erythrocytes, and decreased flow of iron to the liver.

Hemopoietine injections produce a much larger increase in 3 hr erythrocyte Fe^{59} than in plasma iron turnover rate; in fact the former is an exponential function of the latter. This can be explained by assuming that extracts containing hemopoietine not only increase the plasma iron turnover rate—presumably as a consequence of increased consumption of iron by the marrow—but also shorten mean time spent by reticulocytes in the marrow, as in bleeding and hemolytic anemias(12). The marked effect of hemopoietine on 3 hr erythrocyte Fe^{59} is especially clear in the data in Table Ib, where increase in erythrocyte Fe^{59} is about 60-fold, whereas increase in marrow iron consumption is only about 3-fold. Since 30% of injected radioiron was already in red cells by 3 hours, an appreciable flow of iron went to red cells already in circulation or entering it within the 3 hour period.

The significant linear correlation between 24 hour erythrocyte Fe^{59} and 3 hour Fe^{59} shows that study of Fe^{59} distribution at 3 hours gives as good information on erythrocyte Fe^{59} as 24 hr values. It also permits calculation of plasma iron turnover rate and estimation of marrow iron consumption. The latter data indicate how much Hb synthesis has increased, whereas erythrocyte Fe^{59} values alone do not reflect proportionately the changes in marrow iron consumption, presumably because they are influenced by the time spent by the reticulocyte in the marrow. Moreover, this time may vary independently of consumption of iron by Hb synthesizing tissue, and consequently small increases in erythrocyte Fe^{59} 18-24 hrs after tracer injection may not indicate increase in iron uptake by erythroid tissue.

Increases in plasma iron turnover and marrow iron consumption produced by urinary hemopoietine are influenced by plasma iron concentration at the time of Fe^{59} injection (13). In general we have observed that the more potent the preparation of hemopoietine used, the greater is the decrease in plasma iron concentration (Table Ib). This drop of plasma iron could be a reflection of increased marrow consumption, not exactly balanced by input of iron into the plasma from storage or intestinal tract. This accords with the findings that changes in serum iron level, in absence of hemolysis or iron deficiency, reflect changes in magnitude of erythropoiesis(14) and with the correlation between reticulocyte

and marrow response and fall in plasma iron seen in pernicious anemia after treatment with liver extract(15).

The data obtained from quantitative bioassay carried out by the method of Stetten (11) allow a calculation of maximum effect in this type of assay, and also of the dose producing half maximum effects ($D_{½}$ max.). These parameters may prove useful for intercomparison of various fractions obtained in a purification procedure. If an extract showed unchanged maximum effect and decreased $D_{½}$ max. after fractionation, the material eliminated probably did not have a deleterious effect on erythropoiesis, and the decrease in $D_{½}$ max. would measure increase of specific activity of the preparation. However, if some material with deleterious effect on erythropoiesis were eliminated by fractionation the maximum effect might increase.

Our results suggest that in assays of hemopoietine, more adequate information on the state of erythropoiesis can be obtained from studies of Fe^{59} distribution in plasma, liver, marrow and erythrocytes at a time when plasma radioactivity is high, than from determinations of Fe^{59} in erythrocytes 24 hours after tracer injection. The data obtained by measurement of tracer distribution when plasma radioactivity is high allow an estimate of plasma iron turnover and flow of iron to the marrow to be made, and also indicate changes in mean marrow-to-Hb transit time. Such information is useful in assays using normal rate as receptors, for in these the fraction of Fe^{59} in red cells at 24 hours is already high in the controls, and is not very sensitive to changes in plasma iron turnover, whereas plasma iron turnover is a good index of erythropoiesis(16) and can be estimated from the early Fe^{59} distribution studies.

Summary. Fe^{59} distribution in plasma, red cells, femurs and liver is measured at a time (1½ hours in nonfasted, 3 hours in fasted rats) when plasma radioactivity is high. Plasma iron concentration is also measured, and used with the plasma Fe^{59} value to obtain an estimate of plasma iron turnover. This technic is used for assay of urinary hemopoietine from phenylhydrazine treated rabbits and patients with aplastic anemia. Hemopoietine preparations significantly decrease plasma Fe^{59} and increase femoral and red cell Fe^{59}. Fe^{59} in red cells at 24 hours is a linear function of that present at 3 hours. Increase in Fe^{59} in red cells is much larger proportionately than increase in plasma iron turnover, and thus is not a good reflection of the state of erythropoiesis. A dose effect study shows that the reciprocal of the effect, as measured by fraction of plasma iron turned over per hour, is a linear function of the reciprocal of dose of urinary hemopoietine injected.

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