EXHIBIT 8

Studies on Erythropoiesis. III. Factors Controlling Erythropoietin Production. (22910)

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The existence of a plasma factor, known as erythropoietin, that is capable of modifying red blood cell production is firmly established. In 1955, we(1) described a bioassay for this plasma factor in the normal rat. The procedure used the incorporation of Fe^{59} into newly-formed erythrocytes as index of rate of erythropoiesis. More recently, we demonstrated that the hypophysectomized rat is a more sensitive assay animal than the normal one(2,3).

Further studies on the temporal relationship of hypophysectomy to changes in erythropoiesis and on erythropoietic response of the hypophysectomized animal to erythropoietin are the subjects of this paper. In addition, other sensitive assay systems that can be produced by methods designed to decrease erythropoiesis are described.

Materials and methods. A) *Preparation of plasma*: On 3 consecutive days, rabbits or rats were bled by cardiac puncture, and on each of these days, the amount withdrawn approximated 2% of body weight. On alternate days thereafter, blood amounting to about 1.5% of their weight was withdrawn. After the hematocrit was reduced to 25% or less, more blood was withdrawn and heparinized, and plasma was separated and stored at −17°C. This procedure, which has been described in detail(1), was followed for preparation of plasma that we shall refer to as "anemic plasma." Samples from the same lot of anemic plasma were always used in comparative experiments. Plasma, which was prepared similarly from blood of normal, previously unbled rabbits or rats served as control. B) *Assay procedure*: Rats in each experimental group discussed below were given intravenously three 2-cc doses of anemic plasma on successive days. Control animals received normal plasma or saline according to the same schedule. The rate of erythropoiesis was then determined as follows(2): Fe^{59} citrate (2 to 3 μc) was injected into the tail vein of the animal at 3-6 hours following last injection of plasma. Sixteen hours after injection of iron, a 1-cc sample of blood was taken by cardiac puncture. Activity of Fe^{59} in erythrocytes of the sample was determined by counting in a well-type scintillation counter.* The amount of radioactivity in the entire circulating red cell mass was calculated and expressed as per cent of injected dose of Fe^{59} in peripheral red cells. C) *Preparation of recipient animals*: *Hypophysectomized rats.* Hypophysectomized male Sprague-Dawley rats,† 4 or 8 weeks old and weighing 75-90 or 140-160 g, were maintained on diet consisting of milk, fresh vegetables, and Rockland mouse pellets *ad libitum*. The rats were given 3 injections of plasma, and the iron was introduced 6 hours after last injection. Polycythemia was produced in normal, 3-month-old male Sprague-Dawley rats by intraperitoneal injections of washed homologous erythrocytes suspended in saline. Ordinarily, 8 daily injections of 2 cc of red cells were sufficient to elevate the hematocrit from a normal value of about 45% to one between 70 and 75%. The rats were used for assay the day following last injection of red cells. Hematocrit values remained between 70 and 75% throughout the assay so that additional red cell injections were unnecessary. At conclusion of assay procedure, blood volumes, determined by the radiochromium method of Gray and Sterling(4), were 6% of body weight in the polycythemic rat as compared with 5% in the normal rat. *Hyperoxic rats.* Normal, 2-month-old male Sprague-Dawley rats, weighing between 150 and 200 g, were placed in oxygen tent into which flowed a continuous supply of oxygen, maintaining an

* Nancy Wood Counterlab, Chicago, Ill., Model SC-2L-42.

† Obtained from Hormone Assay Laboratories, Chicago, Ill.

atmosphere of 85-95% O_2.‡ After 7 days, the rats were divided randomly into treatment groups of 5 animals each, and the 3-day regimen of plasma injection was initiated. The experiment was concluded on 10th day that the rats were in the oxygen tent. During the 10-day period, the animals were out of the high O_2 atmosphere only for the brief time necessary for injection. *Fasted rats.* Normal, 2-month-old male Sprague-Dawley rats, weighing 175 g, were deprived of all food, but water was supplied *ad libitum*. Radiochromium blood volume determinations after 5 days of starvation gave values of 5% body weight in the starved animal as compared to 5% in controls. At various intervals, as indicated below, rats in randomly-selected groups were injected with Fe^{59}, and the percentage of radioiron incorporated into red blood cells was determined. Other groups were given anemic or normal plasma for 2 days before injection of iron. The rats were weighed at time of sampling. The fast was continued in all cases until conclusion of the assay. *Rats injected with dinitrophenol.* Male Sprague-Dawley rats, weighing between 375 and 400 g, were injected subcutaneously with 5 mg of 2,4-dinitrophenol in a 10-ml volume at pH 8.5 daily for 3 days. One-half of the group received daily intravenous injections of 2 ml of anemic plasma while the other half received saline. Rats in the control group received 10 ml of saline instead of dinitrophenol and anemic plasma or saline as test material. Fe^{59} was administered 2 hours after last injection, and the % uptake of iron was determined as described above. In many experiments, studies of the effects of anemic plasma on elements of peripheral blood were done on either animals used for measurements of uptake of iron, or upon others that were used solely for hematologic investigations. These studies are presented in Paper No. IV of this series.

Results. A. *Effect of age of hypophysectomized rats upon response to anemic plasma.* The rate of decline of erythropoiesis following hypophysectomy in 4-or-8-week-old rats and the responsiveness to anemic plasma at several intervals following surgery were compared. The younger rats were also studied for a longer time to observe changes that occur during equilibration of red cell mass and red cell production. The results are summarized in Fig. 1 and Table I.

‡ Determined by Beckman O_2 analyzer.

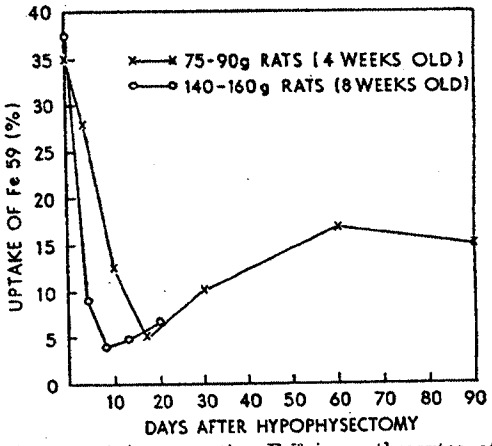

FIG. 1. % incorporation Fe^{59} in erythrocytes of rats hypophysectomized at 4 and 8 wk of age.

In the younger rats, values for uptake of Fe^{59} reached a minimum of 5 to 8% at about 17 days after hypophysectomy. In the older rats, on the other hand, the lowest rate of Fe^{59} uptake, 3 to 5%, was reached at 8 days after removal of the pituitary (Fig. 1). A comparison of responsiveness to anemic plasma of the 2 age groups is presented in Table I.

In the assay of anemic plasma 3 days following hypophysectomy, in the younger rat the ratio of treated to control iron uptake was

TABLE I. Effect of Anemic Plasma on Uptake of Fe^{59} in 4-Wk-Old and 8-Wk-Old Rats at Intervals of 3, 10, 15, 16, and 90 Days after Hypophysectomy.*

Age at operation, wk	Substance	Unoperated control	Days *post-hypophysectomy* when Fe^{59} was inj. 3	10	15	16	90
4	Anemic plasma	45 †	35			16	24
	Control	38	30			8	15
8	Anemic plasma	44.6	17.0	18.1	18		
	Control	37.5	10.1	4.0	5		

* Each interval represents avg for 5 rats/group.
† These figures represent % incorporation of Fe^{59}.

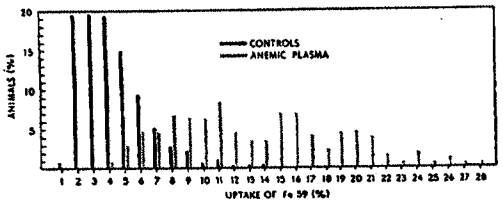

FIG. 2. Distribution of % Fe^{59} incorporation in control hypophysectomized rats and in those treated with anemic plasma.

1.2, while in animals used for assay 16 days after operation, the ratio was 2.0. As would be expected, the relative response to anemic plasma was greater at the time when rate of erythropoiesis was minimal. When the anemic plasma was assayed in older group of rats, the ratios at 3, 10, and 15 days were respectively 1.7, 4.5, and 3.6. These values indicate that in rats of this age, the maximum responsiveness to erythropoietin also occurred at the time of minimum erythropoietic activity, although this was several days earlier than in the case of the 4-week-old animals.

For assay purposes, we use rats that are hypophysectomized when they are 8 weeks old because the minimum rate of erythropoiesis is reached earlier and there is less variability in response than in rats that are subjected to surgery at an earlier age. B. *Variability of assay using hypophysectomized animals as recipients.* To give an indication of variability inherent in the assay procedure described in this and the previous paper(2), we have compiled values for uptake of Fe^{59} for each control hypophysectomized animal (8 weeks old) used in 45 previous experiments. "Control animals" consist of all rats injected with normal plasma or saline or that were uninjected. These controls did not differ significantly from one another. Fig. 2 indicates that 74% of control hypophysectomized animals had Fe^{59} uptake values of 5% or less. In making a similar compilation of the Fe^{59} uptake values for all animals that received anemic plasma (from rabbits or rats), we found that only 0.6% had an Fe^{59} uptake of less than 5% and that 70% had an Fe^{59} uptake exceeding 10%. Since Fig. 2 includes animals from 45 different experiments, many different lots of anemic plasma are represented. This probably accounts for the very broad spread in results.

C. *Polycythemic rats.* In rats with hematocrit values ranging between 70 and 75%, the rate of erythropoiesis, as determined by uptake of Fe^{59}, declined from 32 to 4%. After 8 days of red cell injection, when the rate of new red cell formation was at this minimum, anemic plasma was injected for 3 days. The ratio of treated to control iron uptake values was 6.0 as compared with a ratio of 1.4 for normal rats.

D. *Hyperoxic rats.* Rats subjected to an atmosphere of 85-95% O_2 displayed a diminished rate of erythrocyte production as evidenced by reduction in uptake of Fe^{59} by erythrocytes from control values of 32 to 8% at the 10-day interval. After administration of anemic plasma to these animals, the ratio of treated to control values was 3.0 (normal rats 1.4). Rats maintained in the atmosphere of high O_2 for more than 2 weeks did not survive.

E. *Fasted rats.* The decline in rate of erythropoiesis and increased responsiveness to anemic plasma in starved rats are illustrated in Fig. 3. When the animals lost about one-third of their body weight, their iron uptake values were at a minimum, while their response to injections of anemic plasma was high. The ratios of iron uptake values in treated over controls at 0, 3, 4, 6, and 7 days of fasting were respectively 1.4, 4.7, 5.4, 3.6,

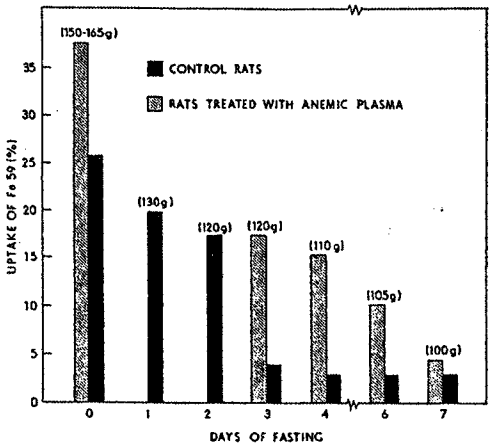

FIG. 3. Effect of duration of starvation on Fe^{59} incorporation and on response to erythropoietin in 8-wk-old rats.

TABLE II. Relative Sensitivity of Assay Preparations to Anemic Plasma in Terms of % Fe^{59} Incorporation.

Recipient*	Plasma inj. Anemic	Control
Polycythemic	24	4
Normal	44	32
High O_2	24	8
Normal	46.5	34
Starved (4 days prior to Fe^{59} admin.)	15	3
Normal	38	27
DNP to normal rat	42	36
Normal	43	22

* Each figure is avg of 5 individual values.

and 1.4. The sharply diminished response in the later stage of starvation probably reflects the over-all deterioration of the animals.

F. *Administration of dinitrophenol* to normal rats resulted in increased rate of erythropoiesis; about 70% greater than that in controls. Animals treated with dinitrophenol showed a response to anemic plasma about 12% greater than that to saline. Untreated animals had a response to anemic plasma that was aboult 100% greater than that to saline. A comparison of the responses to anemic plasma of these variously treated animals is given in Table II.

Discussion. In previous papers(2,3) we presented data indicating that erythropoiesis as measured by incorporation of Fe^{59} or reticulocyte values, declines following hypophysectomy. Administration of anemic plasma to animals in which erythropoiesis is at a minimum elicits an exaggerated response in terms of new red cell formation.

In the present paper we show that rats hypophysectomized at 4 weeks of age, undergo a decline in erythropoiesis that is considerably more gradual than that observed in rats hypophysectomized at 8 weeks of age. The reason for this difference is obscure but certainly deserves further study. The data presented here also indicate that as the new equilibrium between red cell formation and red cell mass is slowly being established, the uptake of Fe^{59} rises from low values found shortly after hypophysectomy to those about midway between pre-operative level and the lowest point. They remain at that level for at least 90 days, (length of our observations).

The following hypothesis was suggested previously in part as an explanation of our data(2,3). After hypophysectomy, the over-all metabolic requirement of the animals drops rapidly to a level that is a fraction of that in the normal animal. There is, therefore, a decrease in the demand for oxygen by the tissues. Since the red cell mass does not decrease significantly within the first 2 or 3 weeks, there exists a relative plethora of erythrocytes, analogous to that in an animal with a transfusion-produced polycythemia. This discrepancy between demand of tissues for O_2 and the amount of O_2 available manifests itself, in some manner, by bringing about a reduction in production of erythropoietin, and as a consequence, erythropoiesis falls to a minimum within a week after hypophysectomy. At this time, administration of anemic plasma rich in erythropoietin would be expected to increase markedly the production of red cells.

Because of the radical initial reduction in erythropoiesis with continuing natural death of red cells, the red cell mass declines slowly. This gradual decrease in O_2-carrying capacity lessens the discrepancy between O_2 supply and demand.

As the plethoric state that was established initially slowly diminishes, the rate of erythropoiesis that had decreased to a minimal value begins to rise until it is able to maintain the red cell mass at a level compatible with the new rate of demand for O_2 by the tissues.

According to this working hypothesis, other experimental conditions that likewise produce a discrepancy between the demand for O_2 and availability of O_2 should also alter the rate of erythropoiesis.

In the experiments with polycythemic rats, the oxygen-carrying capacity of the blood is increased with, probably, no increase in the metabolic requirement for oxygen. This situation is therefore analogous to that in the rat shortly after hypophysectomy and also results in a decreased rate of erythropoiesis and increased responsiveness to anemic plasma.

When animals are subjected to an environ-

ment of 85-95% O_2, there are small but significant increases in the amount of oxygen that is carried(5,6). As in the case of hypophysectomized and polycythemic animals, this condition, by increasing the discrepancy between the supply and demand for O_2, decreases the rate of erythropoiesis and heightens responsiveness to erythropoietin.

Animals subjected to starvation have been shown to have decreased basal metabolic rates very shortly following the onset of the fast (7). In acute starvation, a marked decrease in the tissue demand for oxygen exists without appreciable change in the number of circulating erythrocytes. Thus, a relative plethora of red cells exists in these animals and, like the other preparations discussed above, a decrease in erythropoiesis occurs. Acute caloric deprivation may have an appreciable effect on the synthesis of the plasma factor(s) involved in erythropoiesis. This possibility needs clarification. On the other hand, it is known that protein-deprivation in rats reduces the incorporation of Fe^{59} into the red cells.§

While the situations discussed above all tended to increase the responsiveness of animals to erythropoietin, the rats that were treated with dinitrophenol would be expected to increase their metabolic requirement for O_2 without an immediate, compensating increase in supply of O_2. This is the reverse of the previous cases, and if the hypothesis stated above is valid, the rate of erythropoiesis should increase and the responsiveness to erythropoietin should decrease, as they do. Preliminary experiments with the naturally-occurring metabolic stimulant triiodothyronine gave similar results.

These investigations suggest that formation of erythropoietin and consequently the rate of erythropoiesis are regulated not by the absolute O_2 tension of the blood, but rather by the relationship between O_2 tension of the blood and the oxygen demand by the tissues.

Summary and conclusions. 1. We studied the erythropoietic response to anemic plasma of a variety of experimental conditions in the rat. Rats subjected to hypophysectomy, an atmosphere of high O_2, starvation, and transfusion-induced polycythemia have a decreased rate of erythropoiesis and an exaggerated response to the administration of anemic plasma. 2. Treatment with dinitrophenol increases the rate of erythropoiesis and decreases the response to anemic plasma. 3. These findings are in agreement with the hypothesis that the rate of erythropoiesis is determined by the amount of erythropoietin, the production of which is regulated by the relationship between O_2 supply and demand, not by either factor alone.

§ Bethard, W., and Winsler, R. W., personal communication.

1. Plzak, L. F., Fried, W., Jacobson, L. O., and Bethard, W. F., *J. Lab. Clin. Med.*, 1955, v46, 671.

2. Fried, W., Plzak, L. F., Jacobson, L. O., and Goldwasser, E., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 203.

3. Jacobson, L. O., Plzak, L. F., Fried, W., and Goldwasser, E., *Nature*, 1956, v177, 1240.

4. Gray, S., and Sterling, K., *J. Clin. Invest.*, 1950, v29, 1614.

5. Tinsley, J. C., Jr., Moore, C., Dubach, R., Munnuch, V., and Grinstein, M., *ibid.*, 1949, v28, 1544.

6. Campbell, J. A., *J. Phys.*, 1927, v62, 211.

7. Morgulis, S., *Fasting and Undernutrition*, E. P. Dutton and Co., New York 1923.

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