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Autoradiographic Studies on Iron-59 Turnover by Erythroid Cells in Rat Bone Marrow. (20781)

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From the data on hemoglobin formation in the human bone marrow obtained by Thorell (1) with the aid of absorption microspectroscopy, it is known that "the endocellular synthesis of hemoglobin does not start before the ribose polynucleotide metabolism is finished" and that "the formation of the Fe-porphyrin component and probably also the simultaneous coupling to hemoglobin takes place chiefly during a later maturation phase." Since, from the data of Thorell(1), the ribose polynucleotide metabolism appears to be concluded within the basophilic phase of erythropoiesis, the polychromatic and orthochromatic erythroblasts appear to be the cells in which the Fe-porphyrin and hemoglobin appear first. He has evaluated the hemoglobin content in polychromatic erythroblasts to be 25%, in the orthochromatic 20-25%, and in the erythrocyte 33%, while the basophilic erythroblast either does not contain hemoglobin or the content is not measurable (less than 0.5%). These data, which agree with what is known about hemoglobin formation through the morphology and the staining of immature red cells, do not seem to be substantiated by the isotopic technic, since the rapidity with which the radioiron is taken up by the erythroid cell is so great that it is probable that the earliest stages of the erythroblasts (pro-erythroblasts and basophilic erythroblasts) are also actively involved in the formation of hemoglobin in the red cells. Clinical studies on patients with refractory anemias, showing uptake of iron in the bone marrow, but with little appearing in the peripheral red cells, also suggest that Fe must enter into the immature red cell precursors. This hypothesis can be verified only by using the qualitative procedure of autoradiography of bone marrow smears after radioiron administration. Other technics now available cannot discriminate between the different stages of erythropoiesis. There is no record of such an investigation with iron, although London and coworkers(2), Altman *et al.*(3), and Boyd and associates(4) have employed this technic to demonstrate that glycine labeled with C^{14} or N^{15} is taken up *in vivo* and *in vitro* by the immature red cells of the bone marrow and is incorporated into hemoglobin.

In the present study the stripping film autoradiographic technic, which gives the proper resolution for this purpose, was adopted to detect the first appearance of labeled iron in the earlier stages of erythropoiesis in the rat.

Method. A single dose of 80 μc of Fe-59-labeled ferric chloride in citrated solution was given intraperitoneally to 5 young rats of the Long-Evans strain, weighing about 160-180 g. The animals were then placed under ether narcosis, and sufficient bone marrow to make very thin smears was obtained from the femur or the tibia at specified intervals (½, 1, 3, and 6 hours). In order to obtain hyperplastic and hyperactive bone marrow, two additional animals were bled of half their

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TABLE I. Percentage of Labeled and Nonlabeled Cells in Rat Bone Marrow 6 Hr after Intraperitoneal Administration of 80 Microcuries Fe-59Cl_3.

	Negative	Positive				
	(−)	+	++	+++	++++	Total
Pro-erythroblasts	25	61	11	2	1	75
Basophilic erythroblasts	25	33	32	8	2	75
Polychromatic erythroblasts	12	17	49	20	2	88
Orthochromatic erythroblasts	0	9	38	49	4	100
Red cells	70	21	8	1	0	30

blood volume by heart puncture 4 days before administering 100 μc and 25 μc, respectively. After drying the smears at room temperature, the samples were fixed in pure methanol for 3 to 5 hours. In a darkroom, the smear surface was then covered with 1 cm x 1½ cm stripping film (NTB2 Kodak autoradiographic stripping film) by lifting the slide from beneath the film, which was floating in distilled water (the film must remain in water until thoroughly wet and swollen.) After complete drying in the dark, the slides were stored in a black box for 14 days and then developed with Kodak D19 developer for 3 hours at 20°C. After gentle washing in distilled water or 1% acetic acid (to break the developing), fixing took place in 20% sodium thiosulfate and 5% sodium bisulfite. After careful washing, the preparation was allowed to dry and then examined under the microscope in order to follow the Ag/Br reduction by the radioactivity. The smears were then stained with Giemsa solution through the film for 7 to 8 minutes. Such staining yields a fairly well differentiated preparation; even if the colors of the elements are slightly different from usual, a skillful observer can easily recognize the main characteristics of the cells.

The following characteristics were the most useful in differentiation of the erythroid series: (a) *Pro-erythroblast*: Diameter 12 μ, aspect very like the stem cell, large nucleus with one or two large nucleoli surrounded by a thin superficial layer of nucleolus-associated chromatin, thin cytoplasm of light blue color. (b) *Basophilic erythroblast*: Diameter 10 μ, characteristic chromatin and cytoplasm colors; no nucleus. (c) *Polychromatic erythroblast*: Diameter 8 μ, thicker chromatin and thinner layer of cytoplasm in which it is very difficult to demonstrate the first appearance of hemoglobin; with this technic the cytoplasm is usually uniformly blue. (d) *Orthochromatic erythroblast*: Diameter 6 μ, pycnotic nucleus; with this technic the cytoplasm appears mostly blue-greenish; sometimes it is lightly pink or yellowish.

Results. Since the activity estimation based on grain counting is always subjective, especially when there is, as in our case, a high background, the results will be based on the qualitative impression of the preparations, using quantitative determinations only to differentiate between the active and nonactive cells.

At ½ and one hour after iron administration in the normal rat, there is no definite evidence of activity in the cells, at least after 14 days' exposure of the stripping film and with the radioactivity of the injected material (80 μc). After 3 to 6 hours, radioiron appears in significant amounts in all stages of erythropoiesis as shown by the accompanying tables. From the data presented in Table I, it appears that labeled iron is present in detectable amount as early as in the pro-erythroblastic stage of red cell formation. It is not known if this is connected with hemoglobin formation, but the increasing content of radioiron in the more mature cells (100% of orthochromatic erythroblasts appear active after 6 hours) provides excellent indirect evidence of this assumption. The fact that red cells do not appear to be as active as the immature cells can be attributed to the fact that their population has been diluted by circulating red cells and consequently the results are not comparable.

In the rats rendered anemic by bleeding, the marrow appeared hyperplastic and hyperactive. The qualitative evaluation of the activity of the marrow from these animals is presented in Fig. 1 and Table II. In this

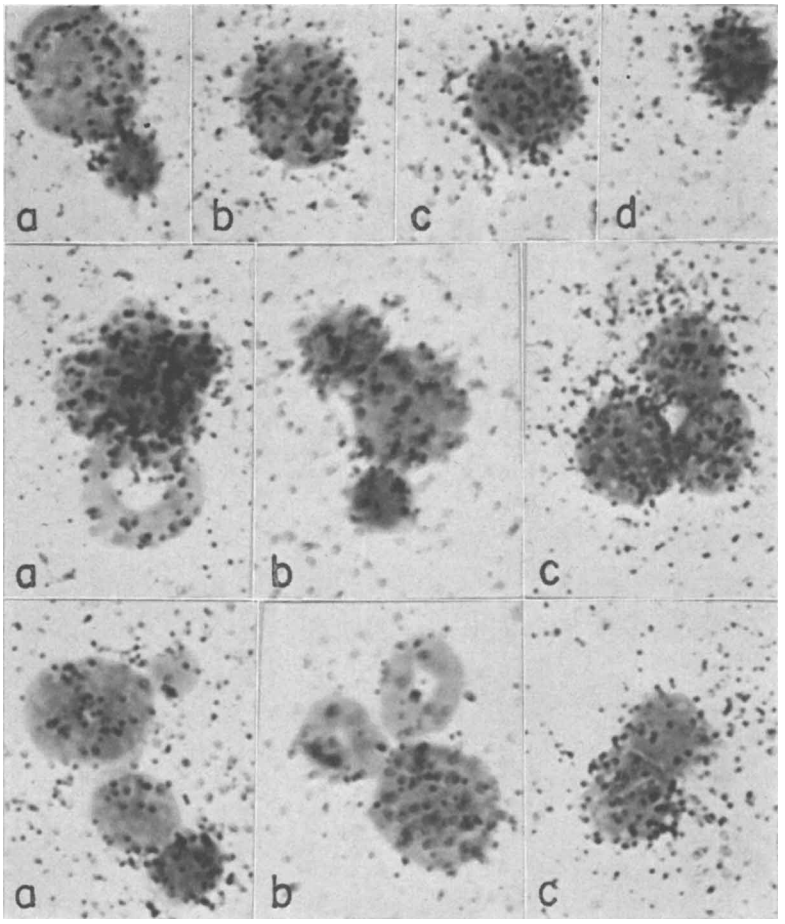

FIG. 1. Autoradiographic pictures of rat bone marrow 3 hr after intraper. inj. of 100 microcuries labeled Fe-59Cl_3 (stripping film autoradiography after 14 days exposure. Staining with Giemsa solution through stripping film, after development. Microphotographs at 1400 ×). In spite of poor differentiation of the element morphology and of the marked background (probably due to old stripping film or to lack of storage in refrigerator after exposure) the activity of labeled immature erythroid cells is evident even in earlier stages of erythropoiesis.
First row (from left to right): Erythroid elements with high activity. In (a) a pro-erythroblast and an orthochromatic erythroblast; (b) a basophilic erythroblast; (c) a polychromatic erythroblast; (d) orthochromatic erythroblast. 2nd row: Groups of erythroid cells with high activity. In (a) group of 4 polychromatic and orthochromatic erythroblast with almost complete darkening of film surface; (b) group of a pro-erythroblast, of a polychromatic and orthochromatic erythroblasts; (c) 3 basophilic elements with marked darkening. 3rd row: Groups of elements with lower activity. In (a) pro-erythroblast, a polychromatic and an orthochromatic erythroblasts with low activity; on the right upper corner an erythrocyte without a definite activity; (b) pro-erythroblast with moderate activity; (c) 2 polychromatic erythroblasts with fairly good activity.

TABLE II. Percentage of Labeled and Nonlabeled Cells in Anemic Rat 3 Hr after Intraper. Admin. of 100 Microcuries Labeled Fe-59Cl_3.

	Negative	Positive				
	(−)	+	++	+++	++++	Total
Pro-erythroblasts	63	32	5	0	0	37
Basophilic erythroblasts	18	55	24	3	0	82
Polychromatic erythroblasts	6	24	48	22	0	94
Orthochromatic erythroblasts	10	55	34	1	0	90
Red cells	93	7	0	0	0	7

case the peak is reached at the polychromatic stage (94%), and higher activity is revealed in the basophilic erythroblasts. These data should be considered in relation to relatively earlier sampling (3 hours), the higher radioiron dosage (100 μc), and the stimulation of bone marrow by previous bleeding.

The present investigation then shows that in the rat and with the doses used, there is a definite turnover of radioiron even by the earliest erythroid elements, namely pro-erythroblasts and basophilic erythroblasts. This was not known before, and it can be assumed as indirect evidence of the earliest stages of hemoglobin formation in the marrow.

Summary. The presence of iron-59 in the earliest stages of the erythroid series of the rat bone marrow was demonstrated by means of stripping film autoradiography after intraperitoneal injection of labeled ferric chlorides. Pro-erythroblasts and basophilic erythroblasts take up a detectable amount of radioiron after 3 to 6 hours. The maximum uptake, however, occurs in the polychromatic and orthochromatic stages.

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A Metabolic Study of Hydrocortisone in Rats. (20782)

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It has been demonstrated that slices of rat liver, kidney and spleen all effectively inactivate cortisone(1). Blood 17-hydroxycorticosteroid levels that have been elevated by administration of a single dose of cortisone or hydrocortisone to humans or experimental animals quickly decline to pretreatment values (2,3). Therefore, it seemed desirable to investigate the participation of the liver, kidney and spleen in the removal of exogenous 17-hydroxycorticosteroids from the blood of the rat *in vivo*.

Materials and methods. Male Sprague-Dawley rats weighing from 190 to 270 g were used without fasting. The removal of organs was carried out through a midline abdominal incision under light ether anesthesia. "Physiological hepatectomy" was performed by a modification of the method of Russell(4). The portal vein, hepatic artery and common bile duct were ligated after securing the gut from pylorus to descending colon in a mass ligature. This operation will be referred to henceforth as hepatectomy. Nephrectomy and splenectomy were accomplished after ligating and cutting the major blood vessels to these organs. The abdominal incision was closed with sutures. Control animals were subjected to laparotomy and a handling of the viscera. It was estimated that hepatectomy removed about 3 ml of blood from the circulation. To evaluate the effect of reduced blood volume upon 17-hydroxycorticosteroid levels, 3 ml of blood were withdrawn from the inferior vena cava in a group of rats through an abdominal incision, and absorbable gelatin (Gelfoam) was applied for hemostasis. Gelfoam was also placed in the peritoneal cavity of control animals for this group. Immediately following each operation, 0.5 ml of a hydrocortisone solution was injected into a tail vein. Solutions were prepared in concentrations of

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