Erythropoiesis and Delta-Aminolevulinic Acid Dehydrase Activity.* (29568)

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There is evidence that one action of the hormone erythropoietin (ESF) is to induce an increase in the number of circulating red cells by causing undifferentiated hematopoietic cells (stem cells) to differentiate into proerythroblasts(1,2,3). Availability of greater numbers of differentiating stem cells would result, after a lag period, in increased numbers of mature erythrocytes. Since once a stem cell has begun to synthesize hemoglobin, it is no longer a stem cell, but a proerythroblast, it has been suggested that erythropoietin may cause stem cell differentiation simply by inducing in these cells, the synthesis of hemoglobin synthesizing enzymes. The enzyme delta-aminolevulinic acid dehydrase (ALA dehydrase) is believed to catalyze the rate-limiting step in the synthesis of heme (4). Previous studies have shown an increase in ALA dehydrase activity in the spleen and bone marrow of erythropoietically active animals(5,6). The present investigation was concerned with further experiments on the effects of erythropoietin and altered conditions of erythropoiesis on the activity of this enzyme.

Methods and results. The ALA dehydrase activity of homogenates and sub-cellular fractions of bone marrow and spleen from adult male Long-Evans rats, and adult female CF-1 mice was determined by a modification of Gibson's method(5). Blown-out rat bone marrow, excised rat liver, and excised rat and mouse spleen were homogenized for 2-3 minutes in a medium containing 0.25 M sucrose and 0.15 M KCl, using a motor driven, Teflon-pestle homogenizer. The preparations were used either as whole homogenates, or as one of two subcellular fractions. The supernatant obtained after sedimentation at 110,000 $\times$ *g* for 60 minutes constituted the "soluble fraction." The "ribosome and soluble fraction" was the supernatant secured after centrifugation at 10,000 $\times$ *g* for 15 minutes.

Each Thunberg tube contained 0.2 ml of the homogenate preparation, 0.8 ml of M/15, pH 6.8 phosphate buffer, 0.1 mMole of glutathione, and, in the side-arm well, 0.1 ml of 0.1 M delta-aminolevulinic acid HCl, neutralized to pH 7. After the one-hour preliminary incubation, the contents of the side-arm were mixed with that of the tubes, and the latter were incubated in a nitrogen atmosphere at 37°C, usually for one hour. Porphobilinogen was estimated using the method and the absorption coefficient of Cookson and Rimington(7). Protein was determined by Lowry's method(8).

Sheep plasma erythropoietin‡ was used in this study, the step 3 preparation having a specific activity of 3.7 units per mg of protein, and the step 4 ESF containing 22.7 units per mg of protein. A portion of the step 4 erythropoietin was inactivated by heating the dry powder over a Bunsen flame until it assumed a uniform brown color, and resuspending the ashed material in saline. Bovine plasma glycoprotein (Cohn fraction V) was obtained from a commercial source.§

In one experiment, the ALA dehydrase activity of the soluble fraction of spleen homogenates from normal, anemic, and polycythemic rats was determined. Samples from each animal were assayed in triplicate. Eight rats were hypertransfused by a single intraperitoneal injection of 10-12 ml of washed homologous red cells suspended in saline (hematocrit about 70%) and sacrificed 5-8 days later. Ten animals were made anemic by two 4-6 ml bleedings, the first performed at 3-5 days, and the second at 30-48 hours

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TABLE I. Hematocrit and Spleen Soluble Fraction ALA Dehydrase Specific Activity of Hypertransfused, Bled, and Normal Rats.

Exp group	No. of animals	% Hematocrit*	ALA dehydrase specific activity†*	Difference from normals t value	Difference from normals p value
Hypertransfused rats	8	70.5 ± 1.99	20.8 ± 3.90	3.09	.01
Bled "	10	30.0 ± 1.00	301.0 ± 34.6	5.65	.001
Normal "	7	46.1 ± .68	79.2 ± 18.5	—	—

* Mean ± $S.E._m$

† mμMole/mg protein/min.

before sacrifice. Seven normal rats served as controls.

In later experiments using normal animals, the soluble fractions from bone marrow and liver homogenates, and the 2 spleen homogenate fractions were assayed for ALA dehydrase activity, when incubated with sheep plasma erythropoietin. An equivalent amount of bovine glycoprotein, ashed erythropoietin, or saline was used as a control. In another assay, the pooled whole spleen homogenate from 2 polycythemic rats (hematocrit—65%) was used. These animals had been injected intraperitoneally with 8 ml of washed homologous red blood cells suspended in saline (hematocrit about 70%) and were sacrificed 5 days later. In this assay, 4 μMoles Na_2ATP and 5 mMoles $MgCl_2$ were added to each Thunberg tube.

The results of the first experiment are shown in Table I. There was a significant 4-fold increase in the ALA dehydrase specific activity of the soluble fraction of spleens from anemic rats as compared to the normal controls. Moreover, the specific activity of spleen soluble fractions from the transfused polycythemic rats was approximately one-quarter that of the controls.

For all groups, each hematocrit value was plotted in Fig. 1 against its respective enzyme specific activity. The latter was found to decrease exponentially with the increase in hematocrit. The coefficient of correlation was —0.895.

Since good correlation between specific activity of a heme-synthesizing enzyme, hematocrit, and the probable level of erythropoiesis was observed, an investigation of the effects of erythropoietin upon ALA dehydrase was undertaken. It was possible that erythropoietin might exert its stimulatory effect by activating previously synthesized enzyme. For this reason, the soluble fractions of rat liver and bone marrow homogenates, and of mouse spleen homogenates were incubated with erythropoietin or control substances, and assayed for ALA dehydrase activity.

The results of these assays are shown in Table II. There was a significant increase in ALA dehydrase activity in tubes incubated with erythropoietin over that of the controls containing glycoprotein in only one out of 7 trials (Exp. 4). On the other hand, significantly higher enzyme activity in the tubes containing the glycoprotein than those containing added ESF, was also seen in one

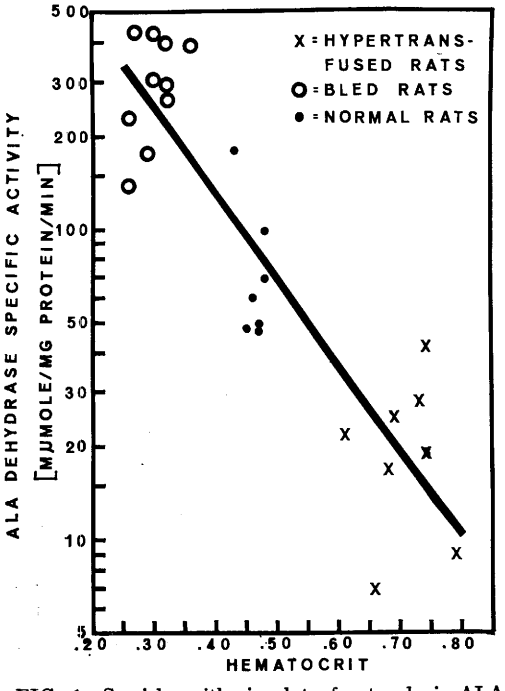

FIG. 1. Semi-logarithmic plot of rat splenic ALA dehydrase specific activity as a function of hematocrit.

TABLE II. ALA Dehydrase Specific Activity of Tissue Homogenates and Fractions Incubated for One Hour with Step 3 (S3) or Step 4 (S4) ESF or Control Substances.

Exp No.	Tissue preparation	mg Protein in added tissue preparation	Added test substance	ALA dehydrase specific activity*†	No. of samples	Difference from controls t value	Difference from controls p value
1	Rat liver soluble fraction‡	12.6	7U S3 ESF	245 ± 22.1	5	2.43	.10
			Ashed ESF	190 ± 17.3	5		
2	Rat marrow soluble fraction§	8.82	2U S3 ESF	103 ± 10.6	8	1.43	.5
			Glycoprotein	75.0± 15.5	8		
3	Rat marrow soluble fraction§	1.47	5U S3 ESF	90.0± 11.4	8	1.85	.1
			Glycoprotein	62.4± 6.6	8		
4	Mouse spleen soluble fraction	2.48	5U S3 ESF	296 ± 11.5	8	4.26	.01
			Glycoprotein	125 ± 43.3	8		
5	Mouse spleen soluble fraction	2.41	5U S3 ESF	428 ± 20.5	8	1.02	.5
			Glycoprotein	376 ± 49.4	8		
6	Rat liver soluble fraction‡	6.28	10U S3 ESF	221 ± 14.9	8	1.64	.5
			Saline	253 ± 19.4	8		
7	Mouse spleen soluble fraction	2.54	5U S3 ESF	223 ± 20.2	8	3.28	.02
			Glycoprotein	302 ± 13.9	8		
8	Mouse spleen ribosome & soluble fraction	2.60	5U S3 ESF	285 ± 7.7	8	16.4	.001
			Glycoprotein	20 ± 7.9	8		
9	Mouse spleen ribosome & soluble fraction	3.22	5U S3 ESF	151 ± 11.6	8	7.48	.001
			Glycoprotein	38 ± 11.6	8		
10	Mouse spleen ribosome & soluble fraction	2.84	10U S4 ESF	225 ± 11.0	6	4.91[1]	.01
			Ashed ESF	259 ± 11.5	6	1.10[2]	.5
			Distilled water	247 ± 10.0	6	.67[1]	.6
11	Hypertransfused rat spleen homogenate	10.51	5U S4 ESF	11 ± .8	6	1.68[1]	.5
			Saline	15 ± 1.4	5	2.25[3]	.1
			Ashed ESF	14 ± 1.5	6	—	—

* $m\mu$Mole/mg protein/min incubation only. † Mean ± S.E.$_m$ ‡ 3 ml total vol, all others, 1.5 ml. § ½ hr

[1] Compared to ashed ESF. [2] Compared to water. [3] Compared to ESF.

assay (Exp. 7). The 5 other runs yielded insignificant results.

There was also the possibility that erythropoietin might act by inducing the synthesis of ALA dehydrase. This effect could be exerted on the ribosomes, or could be exerted on the nucleus, by stimulating the synthesis of messenger RNA. Erythropoietin was incubated with mouse spleen ribosome and soluble fraction preparations, and a polycythemic rat spleen homogenate. These tubes were also assayed for enzyme activity. The results of these experiments are seen in Table II. The ALA dehydrase specific activities of samples incubated with erythropoietin were 12 and 4 times greater, respectively, than that of the controls (glycoprotein) in Exp. 8 and 9. However, the samples containing glycoprotein assayed unusually low. Control specific activities (ashed ESF and distilled water) were slightly higher in Exp. 10 than the ESF specific activity with the ashed ESF value significantly so. The data imply that bovine plasma glycoprotein might have had an inhibitory effect on ALA dehydrase activity, and that ESF exerted no stimulatory influence on enzyme activity.

The polycythemic rat spleen homogenate incubated with ESF (Exp. 11) showed a somewhat lower enzyme specific activity than the ashed ESF and the saline controls. The activities were low in all 3 groups, but consistent with the previous finding of low enzyme values in polycythemic animals. An induction of ALA dehydrase synthesis by erythropoietin thus could not be demonstrated in this type of system.

Discussion. The relationship observed between the various levels of erythropoiesis and enzyme activity was probably caused by the

differences in the relative numbers of erythroblastic cells in the spleens of the 3 experimental groups, *i.e.*, a relatively large number that were actively synthesizing hemoglobin in the bled rats, a smaller proportion in the normal animals, and practically none in the hypertransfused rats. The inability of the sheep plasma ESF to induce either ALA dehydrase synthesis in spleen homogenates and ribosome fractions, or enhance ALA dehydrase activity of spleen, marrow and liver soluble fractions implies that this relation was not due to a direct action of ESF upon this enzyme system. The basic action of ESF is best interpreted as due to its ability to increase the *numbers* of hemoglobin synthesizing cells in the spleen and bone marrow.

Summary. The delta-aminolevulinic acid dehydrase activity of rat and mouse spleen, bone marrow and liver fractions and homogenates was determined. Spleen ALA dehydrase specific activity of normal, polycythemic, and anemic rats was found to decrease exponentially with the increase in hematocrit of the animal. However, sheep plasma erythropoietin, incubated with the tissue fractions and homogenates, did not directly produce an increase in enzyme activity over that of the controls.

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Water Absorption from the Intestine Via Portal and Lymphatic Pathways in Rats.* (29569)

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It is generally accepted that water is absorbed from the intestine via the portal venous system. However, the study of Kim and Bollman(1) suggested the possibility that the intestinal lymphatic system might be another significant pathway of water absorption. To test this possibility Benson *et al*(2) conducted experiments on rats and showed that at least 99% of the water absorbed from small intestine was carried *via* portal venous system.

Lee(3) recently provided contradicting experimental evidence, *in vitro,* showing that the portal system may not be the major route for water absorption. Segments (15-25 cm) of the rat's small intestine were prepared by cannulating the lymphatic and blood vessels. His results showed that 85% of the absorbed water entered the lymphatics and that only 11% was absorbed by the portal vein.

This paper reports experiments with tritium oxide designed to measure the quantities of water absorbed into both the lymphatic and portal channels in the intact rat.

Methods. Twenty-seven adult Sprague-Dawley female rats averaging 200 g body weight were anesthetized with sodium pentobarbital (32.5 mg/kg). Cannulation of the mesenteric lymph duct was done according

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