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Research Article

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Nucleoside Deaminase: an Enzymatic Marker for Stress Erythropoiesis in the Mouse

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ABSTRACT The level of nucleoside deaminase was determined in extracts of mouse tissues obtained during a period of accelerated erythropoiesis induced by hypoxia, hemorrhage, or the injection of phenylhydrazine. Under these conditions a striking (10- to 100-fold) elevation of the enzyme activity occurred in the spleen. Similar results were obtained with the injection of purified erythropoietin. In control animals, only a trace of nucleoside deaminase activity was detected in the blood. During the reticulocyte response which followed erythropoietic stimulation, there was a sharp increase in the blood level of nucleoside deaminase, which rose up to 120 times that of control animals. By differential centrifugation, the enzyme was localized to the reticulocyte-rich fraction. Erythrocyte nucleoside deaminase remained elevated even after the reticulocyte count had fallen to normal in the phenylhydrazine-treated mice or to zero after the cessation of hypoxia. There was a very gradual decline in the enzyme activity in the blood which fell to the barely detectable control levels about 45 days after the initial reticulocyte response, a time period which corresponds to the survival of the mouse red blood cell. The persistence of high levels of nucleoside deaminase for the full life span of a generation of erythrocytes formed during stress, viewed in contrast to the virtual absence of the enzyme from normal erythrocytes of all ages, represents an enzymatic difference between the normal red blood cell and the cell produced under conditions of accelerated erythropoiesis.

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INTRODUCTION

Nucleoside deaminase catalyzes the hydrolytic deamination of cytidine to uridine. Previous investigations in this laboratory had demonstrated a marked increase in the activity of this enzyme in the spleens of mice inoculated with Friend leukemia virus (1).[1] Since the disease induced by the Friend virus is characterized by a massive erythroblastic response in the spleen (2–8), it seemed pertinent to determine whether alterations in nucleoside deaminase levels would occur in rapidly proliferating erythroid tissue of uninfected animals. In the present study, erythropoiesis was stimulated in normal mice and the enzyme activity measured in tissue extracts. The results show that high levels of nucleoside deaminase appear in the erythroid cells which develop during the period of accelerated red blood cell production.

METHODS

Cytidine-2-^{14}C and deoxycytidine-2-^{14}C were purchased from Schwarz Bio Research Inc., Orangeburg, N. Y. Purified human urinary erythropoietin was generously provided by the Erythropoietin Committee, National Heart Institute, National Institutes of Health.

Most experiments were performed with female Swiss-Webster mice weighing 20–25 g. 25 g male Swiss-Webster mice and 20 g female DBA/2 mice were used in one experiment with phenylhydrazine. Female CF-1 mice, 20–25 g, were employed in studies utilizing the hypobaric chamber. Studies on species distribution used 75 g male Sprague-Dawley rats, 2 kg New Zealand white rabbits, and 100 g female H.H. Hartley guinea pigs.

Friend virus inoculation was performed by i.p. injection of a cell-free homogenate obtained from leukemic spleens, as previously described (9).

Erythropoietic stimulation was achieved by four methods. (*a*) Hypoxia was induced by exposure to 0.4 atmospheres of air for 19 hr/day in a hypobaric chamber. (*b*) Erythropoietin (ESF) was administered by i.p. injection. (*c*) Ani-

[1] Malathi, V. G., and R. Silber. In preparation.

mals were bled from the retroorbital plexus under ether anesthesia. Approximately 0.4 ml of blood was withdrawn on each of 3 consecutive days. (*d*) Acute hemolysis was produced by i.p. injection of phenylhydrazine which was prepared immediately before use by dissolving phenylhydrazine HCl (PHZ) in 0.15 M NaCl and adjusting the pH to 7.0. Mice were usually given a single injection of 2 mg of PHZ per animal and then sacrificed at various intervals. Rats were given 2 mg of PHZ on day 0, day 1, and day 3, and sacrificed 4 days after the last injection. Rabbits received 20 mg of PHZ on day 0 and day 1, and were killed on day 5. Guinea pigs were given a single injection of 6 mg of PHZ and sacrificed 4 days later.

Transfusion-induced polycythemia was achieved by the i.p. injection on 2 consecutive days of 1 ml of whole blood which had been obtained from normal isologous donors just before use. The mice were sacrificed 4 days after the second injection. Experiments with splenectomized animals were performed 4 wk after surgery.

Mice were sacrificed by CO_2 narcosis. Heart blood was collected in EDTA (heparin was not used as an anticoagulant because it was found to inhibit the enzyme reaction). Spleens and blood removed from groups of animals killed at a given time were pooled and homogenized with a Teflon Potter-Elvehjem homogenizer in 0.01 M Tris (pH 8.0), 2.0 ml/g of tissue. The homogenate was centrifuged at 11,000 *g* for 20 min at 0–5°C, and the supernatant fluid was used for the determination of enzyme activity. In some experiments, extracts of blood or bone marrow were prepared by freezing and thawing. These extracts had the same enzyme activity as those prepared by homogenization.

In two experiments[2] (effects of hypoxia and hypertransfusion) tissues obtained from individual animals were examined. The spleen was divided into two halves. The blood and one portion of the spleen were homogenized and centrifuged, and the supernatant fluids were assayed for enzyme activity. The pulp was removed from the other portion of the spleen and was uniformly suspended in isologous serum with a rubber-bulbed glass pipette. The hemoglobin-containing nucleated red cells in this suspension were enumerated from smears stained by the benzidine technique (10). The count was expressed as the percentage of total nucleated cells which were benzidine-positive. Benzidine staining does not permit identification of very early erythroid precursors, i.e. proerythroblasts, which lack sufficient amounts of hemoglobin to be detected by the stain (10).

Blood, obtained from mice 5 days after the administration of 2 mg of PHZ, was fractionated by differential centrifugation. The blood was centrifuged in Wintrobe hematocrit tubes at 1800 *g* for 30 min at room temperature. The following fractions were removed with a long 18-gauge needle: the plasma; the buffy coat, which consisted of leukocytes, platelets, and a few erythrocytes; the top one-fourth of the packed red cell column (reticulocyte-rich fraction); and the bottom one-fourth of the packed red cell column (reticulocyte-poor fraction). Contaminating erythrocytes were removed from the buffy coat fraction by osmotic lysis, as previously described (11), and the intact leukocytes and platelets were then washed, collected by centrifugation, and resuspended in a small volume of 0.15 M NaCl. Extracts of these fractions, prepared by freezing and thawing, were assayed for enzyme activity.

Nucleoside deaminase was assayed by measuring the amount of labeled uridine formed by the deamination of 2-^{14}C-labeled cytidine. The assay mixture contained the following: 20 μmoles of Tris-HCl buffer, pH 8.0; 0.05 μCi of cytidine-2-^{14}C (23 mCi/mmole); and the enzyme preparation in a total volume of 40 μl. This was incubated at 37°C for 10–60 min and the reaction terminated by addition of 20 μl of a mixture of unlabeled cytidine, uridine, and uracil (each 0.005 M) in 3 N HCl. Samples to which the acid and reference compounds were added before the enzyme served as blanks. The precipitate was removed by centrifugation, and aliquots of the supernatant fluid were spotted on Whatman No. 1 paper and subjected to ascending chromatography for 16 hr. The solvent system consisted of *n*-butanol: water: formic acid (77: 13: 10). The positions of marker compounds were identified under ultraviolet light and the spots were cut out. The paper was immersed in 15 ml of scintillation fluid (100 ml of toluene, 4 g of PPO, 100 mg of POPOP) and the radioactivity determined in a Beckman liquid scintillation counter, Model LS-100 (Beckman Instruments, Inc., Fullerton, Calif.). A milliunit of enzyme activity is defined as the amount of enzyme which forms 1 mμmole of uridine in 1 min at 37°C, and specific activity is expressed as milliunits per milligram of protein. When spleen extracts were assayed, a small amount of the uridine formed in the reaction was further converted to uracil. Therefore, the radioactivity found in the uracil spot was added to that in the uridine spot in order to calculate the total amount of cytidine which had been deaminated. When deoxycytidine was used as substrate, the corresponding deoxy derivatives were substituted for cytidine and uridine in the acid mixture. All assays were performed in duplicate.

In preliminary experiments with this enzyme assay, it was shown that the product of the reaction corresponded to uridine in three chromatographic systems. Neither PHZ nor EDTA interfered with the enzyme assay. When spleen extracts were used as the enzyme source, the reaction rate was linear only up to 30 min, declining thereafter; for this reason the measurement of splenic enzyme activity was performed with a 10–20 min incubation period. With blood nucleoside deaminase, the reaction rate was linear for at least 60 min, and the assay mixture was incubated for 10–60 min. It was found that the curves illustrating the change in blood nucleoside deaminase after various experimental procedures were very similar whether the specific activity of the enzyme was expressed as milliunits per milligram of protein or as milliunits per milligram of hemoglobin.

Adenosine deaminase was assayed by a modification of the method of Pfrogner (12). Enzyme solution (10–20 μl) was added to 0.5 ml of a 1.75×10^{-4} solution of adenosine in 0.1 M sodium phosphate buffer, pH 7.0. This mixture was incubated at 37°C for 30–60 min and the reaction terminated by addition of 1.0 ml of 0.1 N HCl. Samples to which acid was added before the enzyme served as blanks. The amount of adenosine which was deaminated to inosine was determined by the decrease in optical density at 265 mμ.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (13). Standard techniques were employed for the measurement of hematocrit, hemoglobin concentration, and reticulocyte count.

[2] These experiments were performed with the technical assistance of R. D. Carmichael, Department of Biology, Graduate School of Arts and Science, New York University.

RESULTS

Effect of Friend virus infection. The rapid increase in spleen weight and spleen nucleoside deaminase which occurred after inoculation with Friend virus is shown in Fig. 1. This result confirms earlier observations (1).[1] Also shown in the figure is a 500-fold elevation of nucleoside deaminase activity in the blood, which was associated with the presence of immature nucleated cells. Experiments were next designed to determine whether other stimuli would affect the levels of spleen and blood nucleoside deaminase.

Effect of hypoxia. Mice exposed to reduced barometric pressure were sacrificed at 24-hr intervals and nucleoside deaminase activity was determined in the spleen and blood. The number of erythroid precursors containing hemoglobin was determined with a benzidine stain. The results are shown in Table I, where the values are given for individual animals. Fig. 2 shows the mean values for groups of animals killed at a given time.

On the 2nd day of hypoxia, a 4-fold elevation in the percentage of benzidine-positive cells was observed. These continued to increase until the 8th day when 52% of the cells were benzidine-positive, and declined thereafter. After 21 hr of hypoxia, there was a 6-fold increase in nucleoside deaminase activity in the spleen. It should be noted that this change preceded the rise in benzidine-positive cells. The enzyme levels reached a maximum (12-fold increase) on the 7th day. The activity declined rapidly thereafter, falling to twice the control level by day 14, despite the fact that the animals still remained under hypoxic conditions. The day-to-day fluctuations in enzyme level, observed during the period of increase in nucleoside deaminase activity, are suggestive of a rhythmic erythropoietic response. However, in view of the marked variation of the enzyme levels

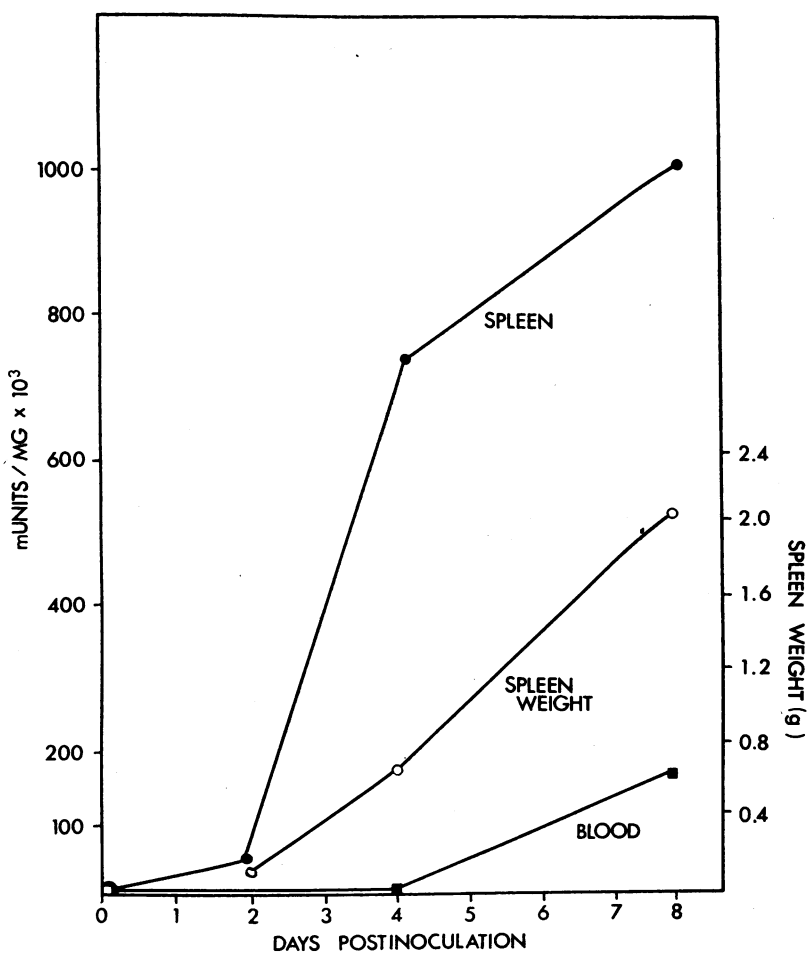

FIGURE 1 Effect of Friend virus inoculation on spleen and blood nucleoside deaminase activity in Swiss mice.

TABLE I

Effect of Hypoxia on Nucleoside Deaminase Activity and on Erythropoietic Parameters

Animal No.	Duration of hypoxia*	Hematocrit	Reticulocytes	Benzidine-positive splenic cells‡	Nucleoside deaminase	
					Spleen	Blood
		vol %	*%*	*%*	*mU/mg × 10³*	
1	Control	52.5	2.5	7.3	9.7	0.56
2	"	54.5	2.0	10.1	10.1	0.35
3	"	52.0	2.0	3.9	9.4	0.44
4	"	53.0	1.0	5.7	12.4	0.49
5	"	54.5	1.5	2.0	14.6	0.34
6	"	50.5	1.2	2.1	13.4	0.34
	Mean ±SE	52.8 ±0.6	1.7 ±0.2	5.2 ±1.3	11.6 ±0.9	0.38 ±0.06
7	21 hr	53.5	3.3	6.9	94.4	0.27
8	"	48.5	2.1	7.5	65.0	0.24
9	"	59.5	3.8	2.7	60.0	0.44
	Mean ±SE	53.8 ±3.2	3.1 ±0.5	5.7 ±1.5	73.1 ±10.7	0.32 ±0.05
10	2 days	60.5	6.7	26.6	59.5	0.54
11	"	62.5	9.3	29.7	71.3	2.2
12	"	60.0	6.5	7.9	60.9	1.7
	Mean ±SE	61.0 ±0.8	7.5 ±0.9	21.4 ±6.8	63.9 ±3.7	1.5 ±0.5
13	3 days	60.0	8.6	19.3	33.7	0.66
14	"	63.0	5.3	16.4	156	3.1
15	"	63.0	7.0	17.3	140	3.0
	Mean ±SE	62.0 ±1.0	7.0 ±1.0	17.7 ±0.9	110 ±38	2.3 ±0.8
16	4 days	62.0	11.7	40.7	129	6.3
17	"	60.0	6.8	18.2	32.7	0.96
18	"	53.5	8.9	27.2	80.9	4.0
	Mean ±SE	58.5 ±2.6	9.1 ±1.4	28.7 ±6.5	80.9 ±27.8	3.7 ±1.5
19	5 days	65.0	11.3	41.6	108	6.8
20	"	61.5	8.9	17.4	50.2	2.2
21	"	62.5	12.0	16.5	32.6	4.8
	Mean ±SE	62.7 ±1.2	10.7 ±0.9	25.2 ±8.2	63.6 ±22.8	4.6 ±1.4
22	6 days	69.0	14.8	28.2	91.0	2.6
23	"	67.5	9.0	32.6	120	3.0
24	"	72.1	14.2	30.9	155	15.6
	Mean ±SE	69.5 ±1.4	12.7 ±1.8	30.6 ±1.3	122 ±18.5	7.1 ±4.3
25	7 days	73.0	11.7	42.5	128	11.4
26	"	70.5	10.8	32.3	77.0	3.7
27	"	72.0	10.8	42.0	236	21.6
	Mean ±SE	71.8 ±0.7	11.1 ±0.3	38.9 ±3.3	147 ±47	12.2 ±5.2
28	8 days	64.0	17.7	50.0	64.7	19.7
29	"	76.0	19.8	54.4	31.8	23.5
30	"	74.5	15.2	50.5	120	31.6
	Mean ±SE	71.5 ±3.8	17.6 ±1.3	51.6 ±1.4	72.2 ±25.7	23.5 ±4.0
31	9 days	75.0	4.6	26.8	15.1	4.5
32	"	70.5	11.9	30.3	26.8	18.1
33	"	78.5	14.8	32.2	46.9	24.2

TABLE I—(*Continued*)

Animal No.	Duration of hypoxia*	Hematocrit	Reticulocytes	Benzidine-positive splenic cells‡	Nucleoside deaminase	
					Spleen	Blood
		vol %	%	%	*mU/mg* × 10^3	
	Mean ±SE	74.7 ±2.3	10.4 ±3.0	29.8 ±1.6	29.6 ±9.3	15.6 ±5.8
34	10 days	57.0	5.4	25.9	63.0	5.7
35	"	72.0	3.1	15.3	23.8	1.3
36	"	75.0	6.8	40.2	63.0	24.5
	Mean ±SE	68.0 ±5.6	5.1 ±1.1	27.1 ±7.2	49.9 ±13.1	10.5 ±7.1
37	14 days	76.0	3.0	19.9	22.9	1.9
38	"	70.5	2.6	16.4	17.6	21.8
39	"	73.0	2.8	13.8	40.1	7.5
	Mean ±SE	73.2 ±1.6	2.8 ±0.1	16.7 ±1.8	26.9 ±6.8	10.4 ±5.9

* CF-1 mice were exposed to 0.4 atmospheres of air for 19 hr/day, except for the 1st day when the duration of exposure was 21 hr. Three animals were sacrificed each day, immediately after interruption of hypoxic stimulation.

‡ The percentage of splenic nucleated cells which are stained by benzidine. Cell counts indicated that during the period of hypoxic stimulation there was no change in the total number of nucleated cells per milligram of spleen. The per cent benzidine-positive nucleated cells therefore reflects the total number of benzidine-positive cells per milligram of spleen.

in individual animals (Table I), the significance of these minor oscillations remains in doubt.

In control animals, only a trace of nucleoside deaminase was detected in the blood. An increase in blood activity was first observed after 2 days of hypoxia. The enzyme activity continued to rise, paralleling the increase in the reticulocyte count. After 8 days, both the number of reticulocytes and the blood enzyme levels began to decline. On the 14th day, when the reticulocyte count was almost back to normal, the enzyme activity in the blood was still markedly elevated.

The persistence of elevated enzyme levels in the blood after discontinuation of the hypoxic stimulus was investigated in the next experiment. Mice were kept in the hypobaric chamber for 8 days and then returned to ambient pressure (Fig. 3). During the 8 days of hypoxia the enzyme activity rapidly increased in the spleen and in the blood. As in the previous experiment, the rise in blood nucleoside deaminase lagged behind the increase in the spleen enzyme activity and paralleled the rise in the reticulocyte count. After the removal of the animals from the hypobaric chamber, the enzyme activity in the spleen returned to near-normal levels. In the blood, however, nucleoside deaminase remained elevated even after the reticulocyte count had fallen to zero. There was a very gradual decline in blood enzyme activity which fell to the barely detectable control levels 45 days after the cessation of the hypoxic stimulus. This is a time period that corresponds to the survival of a mouse red blood cell (14–18). These results indicate that the enzyme activity remains in the blood until the cells produced by the mouse during the period of hypoxic stress have been destroyed. It should be noted that the small reticulocyte response that occurred after the hematocrit had fallen to normal levels was not accompanied by an increase in enzyme activity in the blood. The significance of this will be discussed later.

Effect of ESF. Since the erythropoietic stimulation observed during hypoxia is considered to be mediated by the action of ESF (19–22), the effect of this hormone on nucleoside deaminase activity was next investigated. Mice received 16 injections of ESF over a 40 hr period, as shown in Fig. 4. 10 hr after the start of the ESF treatment, there was a slight increase in enzyme activity in the spleen. A more marked elevation of splenic nucleoside deaminase was seen at 25 hr. The peak activity was reached at 48 hr. Thereafter, the enzyme activity decreased, returning to control level at 120 hr. Blood nucleoside deaminase activity was elevated at 48 hr and reached a maximum at 72 hr, a time when the reticulocyte count was also at its highest level. In control animals, which had been given injections of saline, there was no change in the reticulocyte count or in the enzyme levels.

Effect of hemorrhage. The loss of red cell mass after hemorrhage leads to elevated levels of plasma ESF and to increased rates of red blood cell production (19). The effects of this stimulus were studied in the next experiment. Animals were bled on 3 consecutive days and enzyme levels were measured 2 days after the last

bleeding (Table II). This degree of hemorrhage resulted in a 13-fold increase in splenic nucleoside deaminase activity and in a 23-fold increase in blood enzyme levels.

Effect of PHZ. The potent stimulation of erythropoiesis by the hemolytic action of PHZ is well established (19, 23). This compound was used for the following series of experiments. Groups of animals were killed at 24-hr intervals after an injection of PHZ (Fig. 5). The rapid decline in hematocrit was followed by a striking elevation of nucleoside deaminase activity in the spleen. Enzyme levels increased 9-fold by 24 hr after the administration of PHZ, and by 96 hr the

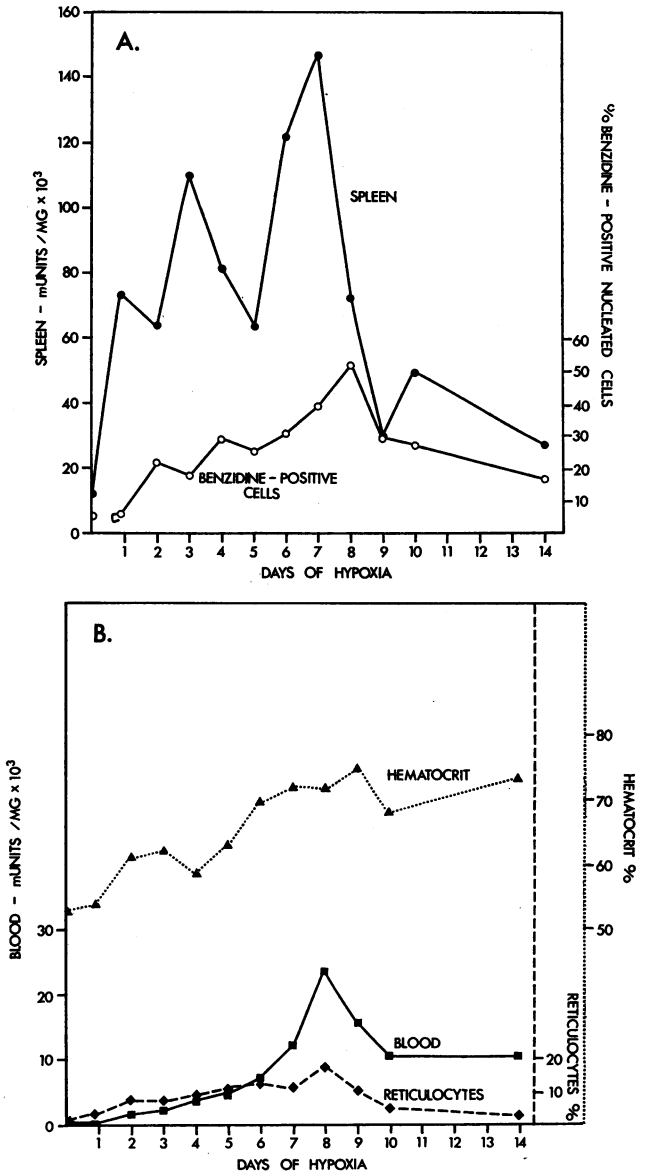

FIGURE 2 Effect of hypoxia on nucleoside deaminase activity and on erythropoietic parameters. CF-1 mice were exposed to 0.4 atmospheres of air for 19 hr/day, except for the 1st day when the duration of exposure was 21 hr. A. Spleen: nucleoside deaminase activity and percentage of benzidine-positive nucleated cells. B. Blood: nucleoside deaminase activity, hematocrit, and reticulocyte count. Each point represents the mean of the values obtained from three animals. The data are presented for the individual animals in Table I.

specific activity had risen to 50 times that of normal spleen. A sharp decrease in enzyme activity occurred thereafter. In the blood, the first significant increase in enzyme levels was observed at 72 hr. At 96 hr, blood nucleoside deaminase activity was 120 times greater than normal. By this time, the blood contained many newly formed red cells, as evidenced by the high reticulocyte count and by the return of the hematocrit to a near-normal level. This experiment was performed using female Swiss mice. Similar responses were obtained when PHZ was given to male Swiss mice of to female DBA/2 mice.

Persistence of enzyme in blood after PHZ-induced hemolysis. After a single injection of PHZ, groups of mice were killed at intervals of up to 48 days (Fig. 6). Nucleoside deaminase activity in the spleen rose to a maximum on the 3rd day, and then returned to normal levels by day 7. The reticulocyte count and the blood enzyme activity reached peak levels on day 5 and then began to decline. By the 10th day, the reticulocyte count had returned to control values. The blood enzyme activity decreased to 25% of its maximum by day 10 and then remained constant for the next 17 days. It slowly declined thereafter. On the 42nd day, the blood activity was still elevated. By the 48th day, it had reached control levels.

Localization of blood nucleoside deaminase. PHZ was administered to a group of mice and blood was collected 5 days later. The following fractions were obtained by differential centrifugation: a reticulocyte-rich red blood cell fraction (the top one-fourth of the packed red cell column); a reticulocyte-poor red blood

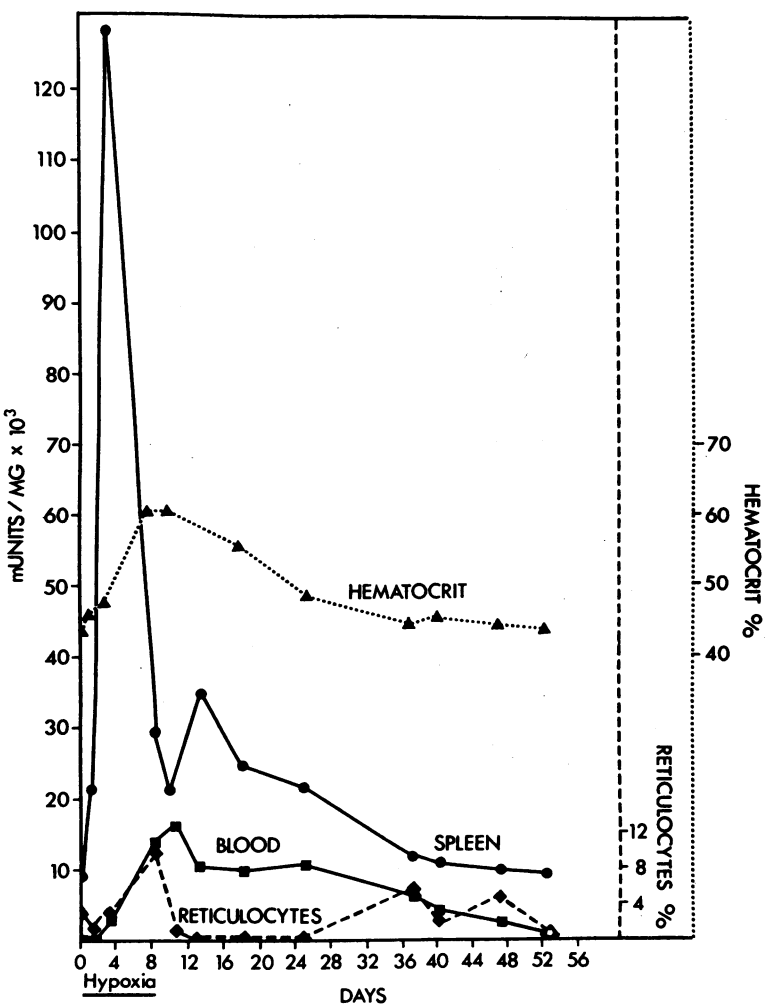

FIGURE 3 Nucleoside deaminase activity during hypoxia and after cessation of the hypoxic stimulus. CF-1 mice were exposed to 0.4 atmospheres of air for 8 days (19 hr/day), and then returned to ambient pressure. Each point represents the value measured in the pooled tissues of three animals.

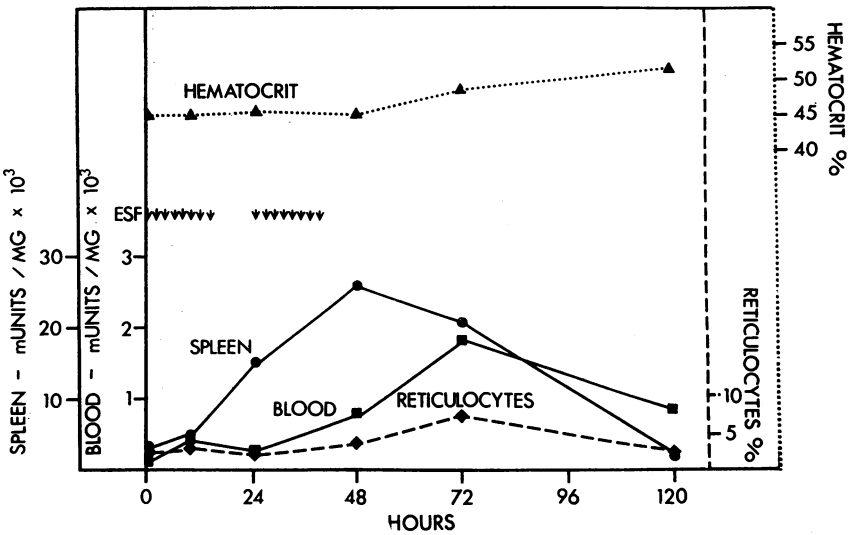

FIGURE 4 Effect of exogenous ESF on spleen and blood nucleoside deaminase activity. The hormone was administered to Swiss mice in a series of i.p. injections. Each injection consisted of 1 U of ESF (arrows).

cell fraction (the bottom one-fourth of the packed red cell column); and a leukocyte-platelet fraction (the buffy coat). As shown in Table III, the reticulocyte-rich fraction contained a 10 times greater level of nucleoside deaminase than did the reticulocyte-poor fraction, and 2.5 times as much activity as the unfractionated blood. The fraction containing the leukocytes and platelets, from which contaminating red cells had been removed by osmotic hemolysis, contained no detectable enzyme activity. The activity in plasma was less than 1% of the activity in erythrocytes.

The results obtained from the fractionation of normal mouse blood are also shown in Table III. The trace level of nucleoside deaminase in whole blood was localized to the erythrocytes, with little difference noted between "top" and "bottom" cells, despite a 5-fold difference in the reticulocyte counts. There was no detectable enzyme activity in leukocytes, platelets, or plasma.

Dose-response to PHZ. A comparison of the effects of different doses of PHZ is shown in Fig. 7. Mice were given single injections ranging from 0 to 3.0 mg of PHZ at the beginning of the experiment. The degree of hemolysis, as indicated by the fall in the hematocrit, was clearly related to the dose of PHZ. The reticulocyte count rose with the administration of increasing amounts of PHZ. In the spleen, there were elevated levels of nucleoside deaminase in all groups; the extent of the increase was commensurate with the dose. In the blood, the enzyme activity was not increased by 0.5 mg of PHZ, although the reticulocyte count had risen to 8%. After 1.0 mg of PHZ, the blood nucleoside deaminase increased 16-fold and the reticulocyte count was 29%. 2.0 mg of PHZ produced a 120-fold elevation in blood enzyme activity and an increase in the reticulocyte count to 40%. Administration of 3.0 mg of PHZ resulted in little further increase in reticulocytes or enzyme activity. Since the enzyme activity appearing in the blood is associated with the reticulocytes, it is apparent from the above data that normal reticulocytes or those formed after mild hemolysis have, at most, only trace levels of the enzyme, reticulocytes formed after moderate hemolysis contain small amounts of the enzyme, and reticulocytes produced after severe hemolysis have high enzyme levels.

Effects of hypertransfusion. In order to determine whether the low levels of splenic nucleoside deaminase in control mice were associated with the erythrocyte precursors present in normal spleen, enzyme levels were measured in animals in which recognizable erythroid activity was suppressed by transfusion-induced

TABLE II
Effect of Hemorrhage on Nucleoside Deaminase Activity

Group	Hematocrit	Reticulocytes	Nucleoside deaminase	
			Spleen	Blood
	vol %	%	*mU/mg* × 10^3	
Control	49.0	1.4	2.9	0.2
Hemorrhage*	42.0	15.2	38.9	4.6

* Swiss mice were bled on 3 consecutive days (a total of 1.2 ml of blood was removed from each animal). Tissues were pooled from five mice 3 days after the last bleeding.

polycythemia (24, 25). In hypertransfused mice, there was no change in the levels of splenic nucleoside deaminase, despite the fact that the number of hemoglobin-containing nucleated cells had diminished to less than 4% of the value found in control animals (Table IV). This indicates that the enzyme activity in normal spleen is not located in the erythroid cells. Hypertransfusion leads to the disappearance of reticulocytes but does not alter the trace enzyme levels normally found in blood. It therefore appears that the reticulocytes are not responsible for the blood nucleoside deaminase activity in control animals.

Effect of PHZ on enzyme activity in other tissues. Table V shows the levels of nucleoside deaminase in various tissues 4 days after the administration of PHZ. Enzyme activity appeared in the bone marrow which normally has undetectable levels. Thus, erythroid stimulation results in elevated nucleoside deaminase activity in the bone marrow as well as in the spleen, both of which are erythropoietic tissues in the mouse. In contrast, there was no appreciable change in kidney or liver, tissues which normally have very high levels of the enzyme.

Effect of PHZ on splenectomized animals. There was a marked increase in blood enzyme activity 4 days after the administration of PHZ to splenectomized animals (Table VI), indicating that the presence of the spleen is not required for the appearance of nucleoside deaminase activity in the blood.

Nucleoside deaminase activity in the blood of newborn mice. Blood was pooled from a litter of newborn Swiss mice, less than 1 day old. Despite a reticulocyte count of 41%, the blood nucleoside deaminase activity (0.2×10^{-3} mU/mg) was no higher than the activity in normal adult mouse blood. It appears that the rapid production of erythrocytes in the fetal mouse was not associated with increased levels of nucleoside deaminase.

Properties of splenic nucleoside deaminase. The normally occurring and the PHZ-induced enzyme were found to have similar properties. The reaction rate was linear for 30 min, and the velocity was proportional to the protein concentration. A broad pH response was observed with an optimum between 5.0 and 7.0. Deoxy-

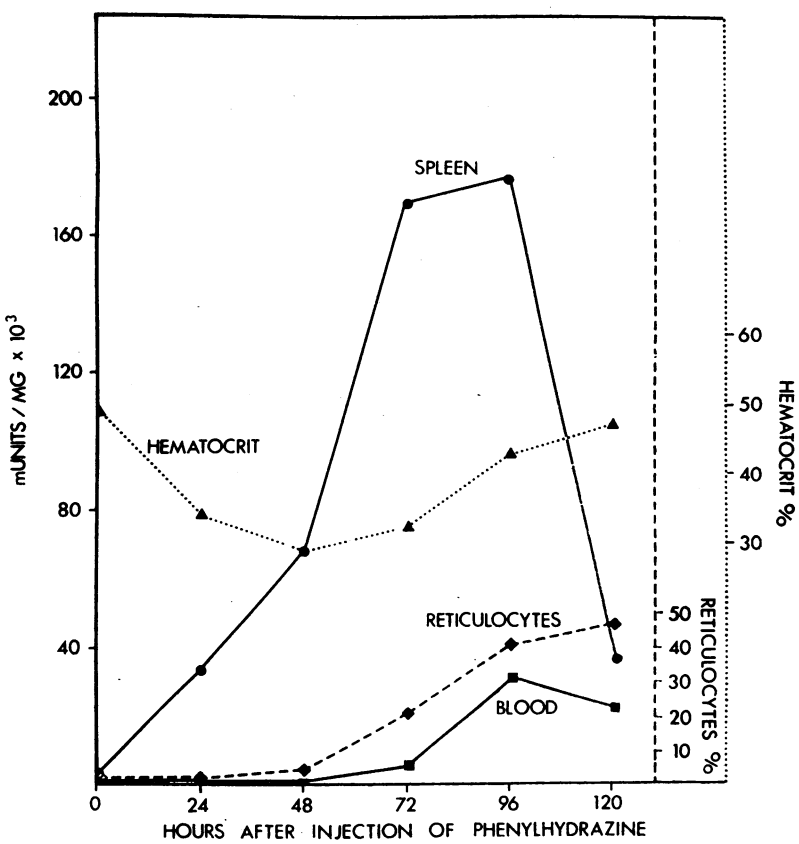

FIGURE 5 Effect of PHZ-induced hemolysis on spleen and blood nucleoside deaminase. Swiss mice received a single i.p. injection of 2 mg of PHZ at zero time. Each point represents a group of three animals.

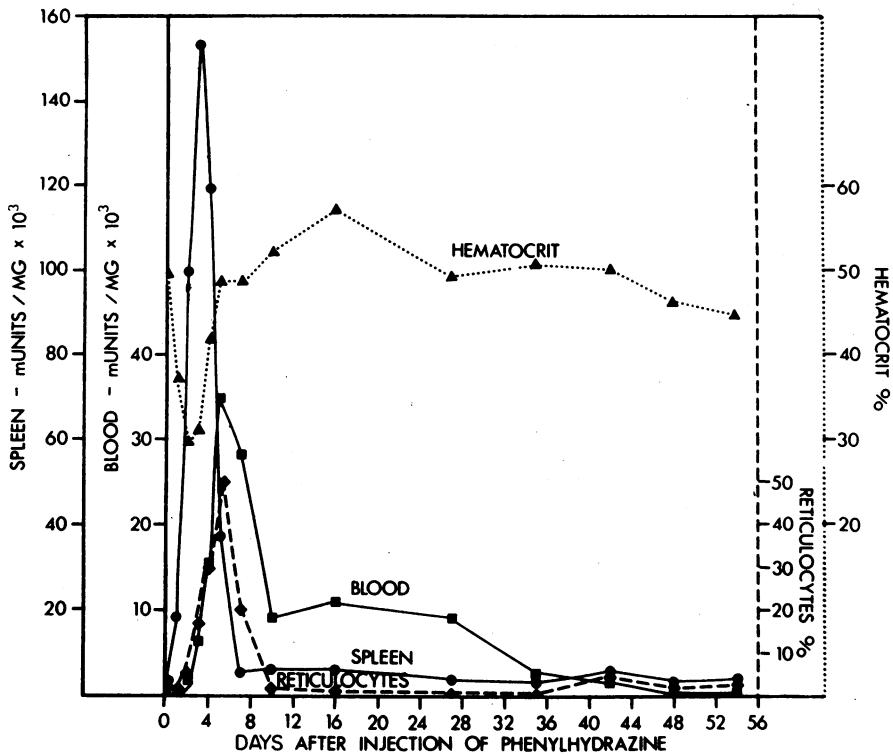

FIGURE 6 Persistence of elevated levels of nucleoside deaminase after PHZ-induced hemolysis. Swiss mice received a single injection of 2 mg of PHZ at zero time. Each point represents a group of three animals.

cytidine was deaminated at 60% the rate of cytidine when both substrates were present at 0.1 mM.

In an experiment in which equal amounts of splenic extracts from normal and PHZ-treated mice were incubated together, the specific activity of the mixture was equal to the activity predicted from the average of the two extracts. This would suggest that the low activity in normal spleen was not due to the presence of an inhibitor and, conversely, the high enzyme level in PHZ-stimulated spleen was not due to the presence of an activator.

Effect of PHZ on adenosine deaminase activity in the spleen. Adenosine deaminase, which catalyzes the hydrolytic deamination of adenosine to inosine, may be considered a counterpart of nucleoside deaminase in purine metabolism. In contrast to nucleoside deaminase, high levels of adenosine deaminase were found in normal spleen (67.6 mU/mg). No increase in splenic adenosine deaminase was observed after the stimulation of erythropoiesis by PHZ.

Lack of effect of erythropoietic stress on nucleoside deaminase activity in other species. No increase in nucleoside deaminase was observed in the spleen or blood of the rat, rabbit, or guinea pig after the administration of PHZ despite high reticulocyte counts. In normal man, there was a high level of nucleoside deaminase in blood. This was localized almost entirely to the granulocytes (26) and no activity was found in erythrocytes. In a study of five patients whose reticulocyte counts ranged from 6 to 20%, no activity was

TABLE III
Localization of Nucleoside Deaminase Activity in Blood

Fraction	Reticulocytes		Nucleoside deaminase	
	PHZ	Normal	PHZ	Normal
	%		*mU/mg of hemoglobin* × 10^3	
Whole blood	45.5	1.4	20.8	0.29
"Top" red cells	81.5	2.6	51.0	0.34
"Bottom" red cells	5.0	0.4	5.2	0.25

Blood, collected from Swiss mice 5 days after the administration of 2 mg of PHZ or from normal Swiss mice, was fractionated by differential centrifugation. The "top" red cell fraction represents the upper one-fourth of the packed red cell column. The "bottom" red cell fraction represents the lower one-fourth of the packed red cell column.

detected in the red cells. The significance of the failure to observe an increase in nucleoside deaminase activity during stress erythropoiesis in species other than the mouse will be discussed below.

DISCUSSION

This report describes an enzymatic difference between normal red blood cells and cells formed during accelerated erythropoiesis in the mouse. Elevated levels of

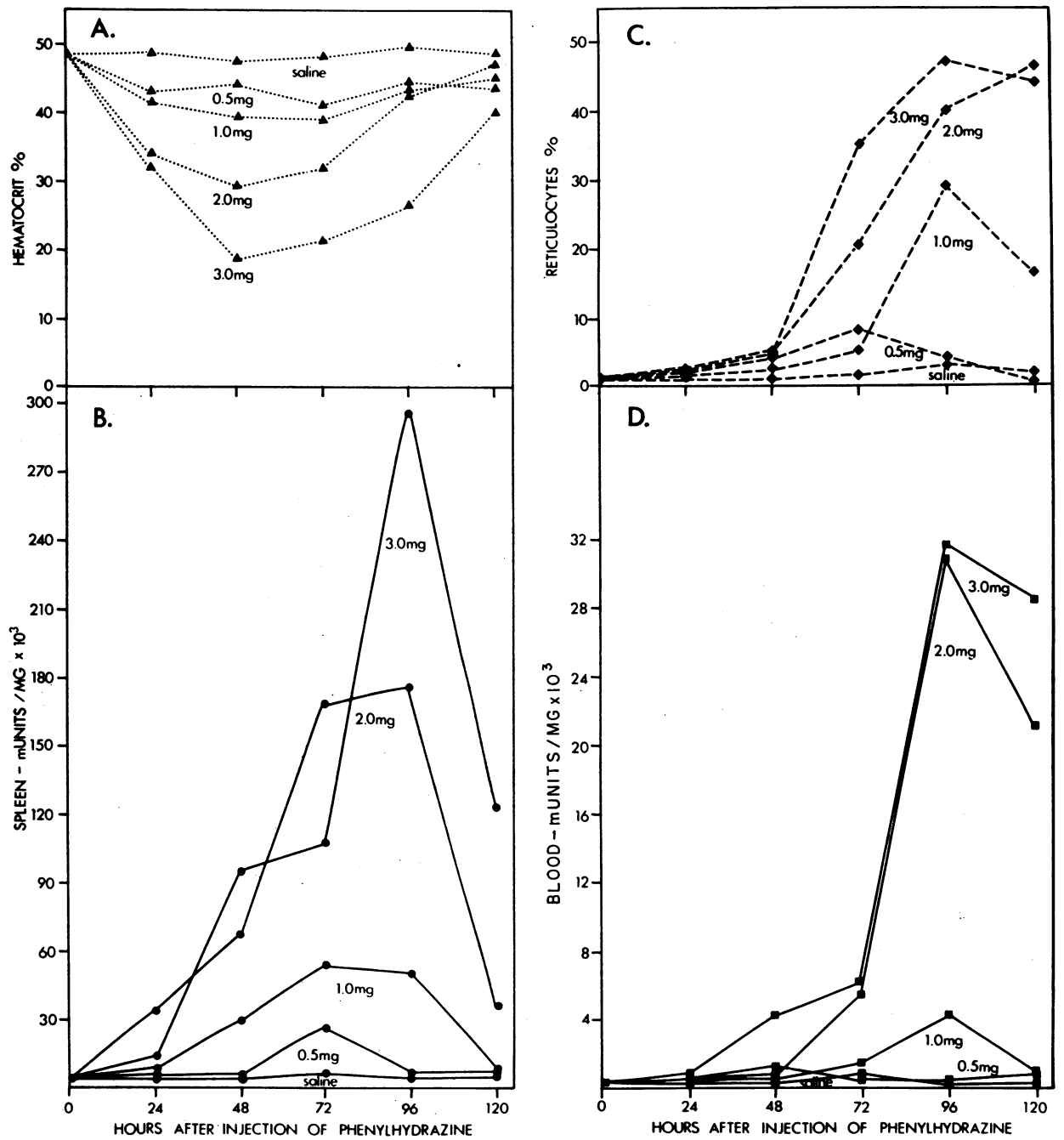

FIGURE 7 Effect of different doses of PHZ on hematocrit, reticulocyte count, and nucleoside deaminase activity. Swiss mice were given a single i.p. injection, at zero time, consisting of 0, 0.5, 1.0, 2.0, or 3.0 mg of PHZ. A. Hematocrit. B. Spleen nucleoside deaminase activity. C. Reticulocyte count. D. Blood nucleoside deaminase activity. Each point represents a group of three animals.

TABLE IV

Effect of Hypertransfusion on Nucleoside Deaminase Activity and on Erythropoietic Parameters

Group	Hematocrit	Reticulocytes	Benzidine-positive splenic cells	Nucleoside deaminase	
				Spleen	Blood
	vol %	*%*	*%*	*mU/mg × 10³*	
CF-1 control	41.7 ±0.3	3.5 ±1.0	14.7 ±6.3	20.8 ±6.6	0.33 ±0.07
CF-1 transfused	59.7 ±0.2	0.00	0.6 ±0.3	20.6 ±3.8	0.53 ±0.09
Swiss control	43.4 ±0.7	0.3 ±0.1	5.3 ±2.4	3.2 ±0.4	0.45 ±0.13
Swiss transfused	59.2 ±1.0	0.00	0.1 ±0.1	4.4 ±1.2	0.44 ±0.11

Polycythemia was produced in Swiss and CF-1 mice by the i.p. injection of 1 ml of isologous blood on 2 consecutive days. The animals were sacrificed 4 days after the second injection. Each group represents three animals. The results are expressed as the mean ±SE.

nucleoside deaminase appear in the spleen and blood after the stimulation of erythropoiesis by reduced barometric pressure, hemorrhage, phenylhydrazine (PHZ)-induced hemolysis, or by the administration of erythropoietin (ESF). Increased enzyme activity is first noted in the spleen as erythroid differentiation is initiated. This is followed by the appearance of the enzyme in the blood when the newly formed reticulocytes enter the circulation. The blood nucleoside deaminase activity is associated with the reticulocyte-rich red blood cell fraction from which the plasma, leukocytes, and platelets have been removed. Enzyme levels remain elevated as the reticulocytes develop into mature erythrocytes and persist for the life span of the cells.

Normal spleen has a low but measurable level of nucleoside deaminase. Hypertransfusion which suppresses almost all of the erythropoietic activity in the spleen has no effect on the enzyme levels (Table IV). These data suggest that the enzyme activity in normal spleen is present in cell types other than those of the erythroid series. The fact that only barely detectable nucleoside deaminase activity is found in the blood of control animals indicates that a population of normal red blood cells contains, at most, trace levels of the enzyme.

TABLE V

Effect of PHZ on the Levels of Nucleoside Deaminase in Various Tissues of the Mouse

Tissue	Nucleoside deaminase	
	Control	PHZ-treated*
	mU/mg × 10³	
Spleen	1.4	113
Blood	0.3	30.6
Bone marrow	0	11.4
Kidney	9680	9050
Liver	528	455

* Tissues were obtained from Swiss mice 4 days after the administration of 2 mg of PHZ.

From these studies, it appears that nucleoside deaminase is not associated with normal erythroid cells at any stage of development.

An increase in spleen enzyme activity is observed within 10–24 hr of exposure to various erythropoietic stimuli. The short duration of this interval suggests that the enzyme first appears in the very early erythrocyte precursors which develop in response to the sudden demand for red blood cells. Elevation of splenic nucleoside deaminase is noted after 21 hr of hypoxia, preceding a change in the number of benzidine-positive cells (Fig. 2). It therefore appears that the enzyme is associated with a more immature cell than the erythroblast which is the earliest benzidine-positive cell (10). Other studies (24, 27, 28) have shown that after the administration of ESF to polycythemic mice proerythroblasts appear in the spleen within 12–24 hr, while erythroblasts are not seen until the 2nd day. Consequently, the increased nucleoside deaminase activity in animals rendered hypoxic for 21 hr is probably located in the proerythroblasts.

During erythropoietic stimulation, erythroblasts appear in much greater numbers and at a later time than proerythroblasts (24, 25, 28, 29). After 21 hr of hypoxia, when the erythroblast count is 6%, nucleoside deaminase activity is 50% of the peak enzyme levels reached on the

TABLE VI

Effect of PHZ on Splenectomized Mice

Group	Hematocrit	Reticulocytes	Blood nucleoside deaminase
	vol %	*%*	*mU/mg × 10³*
Control	42.0	2.0	1.3
PHZ-treated	31.0	21.0	11.2

Splenectomized Swiss mice were divided into two groups (five animals in each). One group served as control. The other group received 2 mg of PHZ. The animals were sacrificed 4 days after the PHZ treatment.

7th day of hypoxia, when the erythroblast count has increased to 39%. Since the number of erythroblasts on day 7 is likely to be much greater than twice the number of proerythroblasts on day 1 (24, 25, 28, 29), it would seem that the proerythroblasts contain more enzyme activity per cell than the erythroblasts. This concept is also supported by the observation that the splenic enzyme levels in hypoxic animals rapidly decline after day 7 even though a maximum number of erythroblasts is seen on day 8 (52%). The loss of enzyme activity in the late erythroid cells could be due to dilution of the enzyme by cell division or to inactivation of the enzyme. Despite the reduction in nucleoside deaminase levels during maturation, the erythrocytes which are formed under these conditions still contain significant enzyme activity.

Additional evidence for the appearance of high levels of nucleoside deaminase in the early cells of the erythroid series is provided by the experiments in which erythropoiesis is stimulated by PHZ or by exogenous ESF. A slight increase in splenic enzyme activity occurs 10 hr after the first dose of ESF is administered (Fig. 4). At 25 hr, the enzyme levels are more than 50% of the peak activity seen at 48 hr. After the injection of PHZ (Fig. 5), nucleoside deaminase activity is elevated at 24 hr, but is only 20% of the maximum activity reached at 96 hr. This relatively small response observed at 24 hr is probably due to the delay in achieving the highest level of erythroid stimulation since the hemolytic process is not complete until the 48th hr, when the hematocrit has fallen to its lowest value.

Nucleoside deaminase activity appears in the blood during the reticulocyte response which occurs under erythropoietic stress. After the cessation of the stimulus by removal of hypoxic animals from the hypobaric chamber or when the hematocrit has returned to normal in PHZ-treated mice, enzyme activity remains elevated despite the fall in the reticulocyte count to zero or to control values, respectively (Figs. 3 and 6). Blood nucleoside deaminase diminishes, thereafter, but does not reach the barely detectable control levels until 45 days after the discontinuation of the erythroid stimulation, a time period corresponding to the life span of a mouse red blood cell (14–18). In posthypoxic animals, the decline in enzyme activity is very gradual. This could represent a random destruction of red cells containing the enzyme, or alternatively, a progressive loss of enzyme activity as the cells age. In PHZ-treated animals, 75% of the enzyme activity is lost during the first few days. This rapid decrease in enzyme activity is consistent with the finding that many of the cells formed during PHZ-induced erythropoiesis are destroyed shortly after their appearance in peripheral blood (30–34). After this rapid loss of enzyme activity, there is a gradual decline in the remaining blood enzyme until control levels are reached by the 48th day. These results indicate that at least some of the cells produced during the interval of erythropoietic stress survive for about 45 days and that nucleoside deaminase activity persists in these "stress" erythrocytes.

The enzyme activity in erythroid cells is related to the degree of erythropoietic stress. Mild stimulation, produced by exogenous ESF or by low doses of PHZ, results in a small increase in splenic nucleoside deaminase. A much greater elevation of enzyme activity is observed during the intense erythroid stimulation produced by hypoxia or by large doses of PHZ. A comparison between the blood enzyme levels and the reticulocyte counts, after the administration of different doses of PHZ (Fig. 7), indicates that the enzyme activity per cell is greater in reticulocytes formed during severe stress (2.0 or 3.0 mg of PHZ) than in those produced during moderate stress (1.0 mg of PHZ). Normal reticulocytes and those appearing after very mild hemolysis do not contain detectable levels of nucleoside deaminase. This is shown by the finding that blood contains the same trace enzyme levels when the reticulocyte count is 0.0% (hypertransfused animals), 1–2% (control animals), or 8% (animals given 0.5 mg of PHZ). Furthermore, in posthypoxic mice, a rebound reticulocytosis (6%) occurs when the hematocrit has fallen to normal levels which is not accompanied by an increase in spleen or blood enzyme activity (Fig. 3).

Previous studies have shown that the activities of many enzymes increase in red cells after erythropoietic stimulation (35–45). These changes are the result of the appearance in the circulation of large numbers of young erythrocytes which contain higher enzyme levels than the older cells (35, 36, 40, 42, 43, 46–50). The enzyme activities decline to normal as the red cells produced under stress age (38–41, 44). In contrast, nucleoside deaminase remains elevated as the cohort of "stress" erythrocytes grow older, indicating that the appearance of this enzyme after erythroid stimulation is not the result of a shift in the age distribution of the red cell population but represents a biochemical difference between normal red cells and cells produced under conditions of accelerated erythropoiesis.

Red blood cells formed under erythropoietic stress differ from normal red cells in several respects. Erythrocytes produced in rats, rabbits, and dogs after acute blood loss or PHZ-induced hemolysis are larger than normal red cells and have a greater mean corpuscular hemoglobin content (31, 34, 51–59). It has been suggested that these macrocytes are produced as the result of a skipped mitosis during the maturation of the erythron (31, 52–54). Furthermore, the cells produced in response to anemia have been shown by cohort labeling

to have a markedly shortened life span (30–34, 51, 56–58, 60–63). Hillman and Giblett (64) have demonstrated an alteration in a human red cell membrane antigen that is associated with marrow stress. The present investigation offers the first evidence for an enzymatic difference between the normal red cell and the "stress" erythrocyte.

It is of interest that nucleoside deaminase activity is not elevated in the blood of neonatal mice. The marked reticulocytosis in these animals and the fact that the red cells are macrocytic (65) suggest that the erythrocytes formed during the fetal period are similar to the "stress" erythrocytes which develop in the adult after erythroid stimulation. The failure to find increased levels of nucleoside deaminase in the red cells of newborn mice may be analogous to the observation that many enzyme activities normally present in adult animals are not found in neonates and appear only during postnatal development (66, 67). Camiener and Smith (68) demonstrated that nucleoside deaminase activity which is very high in adult human liver is not detectable in prenatal liver.

In evaluating the physiologic significance of the appearance of nucleoside deaminase during stress erythropoiesis, two possible functions for this enzyme in nucleic acid metabolism must be considered. One of these is that it might operate in a "salvage" pathway involved in the formation of nucleoside triphosphates for DNA or RNA synthesis (69). Cytidine and deoxycytidine, derived from the breakdown of nucleic acids, would be deaminated to uridine and deoxyuridine and then converted to uridine triphosphate (UTP) and thymidine triphosphate (TTP) for reutilization in nucleic acid biosynthesis. Alternatively, nucleoside deaminase may function in a degradative pathway (70). All of the purine and pyrimidine nucleosides, with the exception of cytidine and deoxycytidine, can be further metabolized through the actions of the nucleoside phosphorylases, which split ribose off the nucleoside (71–74). The inability of cytidine and deoxycytidine to undergo this phosphorolytic reaction makes them inert compounds in further catabolic pathways, unless they are first deaminated to uridine or deoxyuridine by nucleoside deaminase. The uridine nucleosides can then be further catabolized to β-alanine (75).

Although sufficient information is not available for deciding whether nucleoside deaminase functions in a biosynthetic or in a catabolic process, a hypothesis can be offered for a physiologic role of this enzyme in the degradation of nucleic acids in the "stress" erythroid cell. The maturation of the erythron is accompanied by a progressive decrease in cell RNA concentration (76–78). This occurs by the breakdown of RNA to low molecular weight products (76, 79). During erythropoietic stress, erythroid development is characterized by a decreased marrow or spleen transit time (29, 31, 80–82), which may be the result of shortened cell-cycle times (29, 81, 83) or of a reduction in the number of mitotic divisions (31, 52–54) or both (84). The loss of RNA might therefore occur during a shorter time period than in normal erythropoiesis. Under these conditions of accelerated nucleic acid breakdown, cytidine would accumulate in the cell, perhaps overloading the normal disposal mechanisms. The induction of nucleoside deaminase could provide a pathway for the rapid elimination of cytidine. In normal erythroid cells which appear to lack nucleoside deaminase activity, there are presumably other mechanisms for handling the cytidine formed from the catabolism of RNA. In this respect, Bertles and Beck (76) have shown that in rabbit reticulocytes aging in vitro cytidine produced by the degradation of nucleic acids diffuses out of the cells.

Elevated levels of nucleoside deaminase are found in "stress" erythrocytes of the mouse but not in those of the rabbit, rat, guinea pig, or man. This may be a reflection of the marked species differences observed in tissue distribution of nucleoside deaminase activity (68, 85, 86) rather than an indication that the mechanism of stress erythropoiesis in the mouse is different than in other animals. Stimulation of red blood cell production may lead to alterations in the maturation pattern of the erythron, e.g., a shortened cell-cycle time or a skipped mitosis, which are the same in all species, but the biochemical expression of these changes may vary among different animals. It is likely that there are alterations in other enzyme activities which occur during accelerated erythropoiesis in the mouse and in other species. Wilmanns, Sauer, and Gelinsky (87) recently reported that the reticulocyte response, which follows erythroid stimulation in man, is associated with an increased activity of formyltetrahydrofolic acid synthetase in blood, which does not decline as the reticulocyte counts return to normal. Their data strongly suggest that the increase in enzyme activity is not merely due to a shift in the age distribution of the cells but represents a difference between normal and "stress" erythrocytes. It may be that man responds to marrow stress as does the mouse with the production of metabolically altered red blood cells.

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