Changes in Activities of Some Enzymes during *in Vivo* Aging of Mouse Erythrocytes* (32872)

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It has been claimed that enzyme activities decline as the erythrocyte ages. Two approaches for obtaining cells of varying ages have been generally employed. One approach is that of *in vitro* separation of a mixed population of cells by exploiting the lower density or the greater osmotic resistance of young red cells. With these fast and relatively simple techniques, red cell populations can be separated into fractions with a mean age lower, or higher, than the original population. Unfortunately there is not a precise relationship between red cell age and either density (1), or osmotic fragility (2). An alternate approach to the problem is by use of the Ashby technique (3) in which donor's erythrocytes are allowed to age in the recipient's circulation and are separated by selective agglutination at successive intervals. This technique is more complicated and time-consuming and suffers from possible immunological differences between donor and recipient. A third approach has recently been introduced, that of suppressing erythropoiesis by continued administration of actinomycin D (4). The resulting red cell population thus becomes progressively older since no new cells enter the circulation, while those cells already present continue to age. Cessation of the drug administration is followed by a marked reticulocytosis, during which time the influence of reticulocytes on enzymes activity can readily be observed. The present study utilizes this drug technique to study changes in the activities of some enzymes of the mouse erythrocyte during *in vivo* aging and during the subsequent period of reticulocytosis.

Materials and Methods. Arrest of erythropoiesis in mice was achieved as described by Reismann and Ito (4). Solutions of actinomycin D (Merck, Sharp, and Dohme), 5 μg/ml in 0.85% saline, were freshly prepared every 4 days. Adult female random bred Swiss mice were kept on Rockland Rat Mouse Diet and for 35 days injected subcutaneously with either 120 μg/kg on alternate days or 60 μg/kg daily. After this time most of the animals were allowed to recover but in some mice, the drug was continued through day 49. At intervals of 3–4 days throughout the experiment, several mice were sacrificed and blood obtained by heart puncture using heparin as an anticoagulant. The red cells were washed 3 times with 0.85% saline and the hematocrit of the suspension adjusted to 10%. Red cell counts were performed on the Coulter Counter B. Reticulocytes were counted with the new methylene blue N method. Density distribution curves were made as described by Danon and Marikovsky (5). Glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD) were assayed by the method of Bishop (6). Glutamic-oxalacetic transaminase (GOT) was assayed by the method of Henry (7) except that the red cells were first hemolyzed in 0.01% saponin solution, and wherever possible, reagents were combined beforehand and stored frozen, the Tris buffer system being substituted for phosphate. Enzyme activities were expressed as megamolecules (millions of molecules)/min per red cell. The calculations were based on the number of NADPH or NADH molecules generated or used per molecule of substrate, e.g., one each for the G-6-PD and 6-PGD reactions and two in the GOT reaction. All assays were performed at 37°C.

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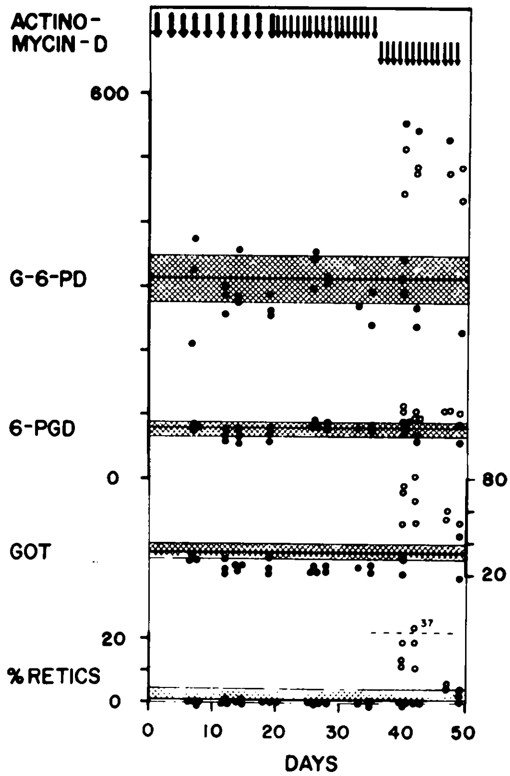

FIG. 1. Enzyme levels and reticulocyte counts during actinomycin D treatment (●). On day 35, the drug was discontinued in one group of the animals (○), but continued in the others. The normal ranges (mean ± 1 SD), derived from 19 animals, is indicated by the shaded areas. Actinomycin D injections are indicated by heavy arrows (120 μg/kg) or by light arrows (60 μg/kg). Each dot or circle represents the mean value of 2 or more assays in one sacrificed animal. Enzyme activities are expressed as megamolecules/min per red cell, at 37°C.

Results. Drug effects. Actinomycin D injections of 120 μg/kg on alternate days caused a marked suppression of erythropoiesis but occasionally some reticulocytes were still found. Therefore, the mode of drug administration was altered to daily injections of 60 μg/kg and subsequently, erythropoiesis was completely suppressed, as judged by the total absence of reticulocytes in the peripheral blood. The observations are in agreement with Reismann and Ito (4). Aside from the progressively lowering of the hemoglobin, the treated mice showed little symptoms of toxicity during the first 4 weeks, but after that time they lost weight (up to 40% in the most extreme cases), were pale and less active, and had a dry fur which sometimes fell off in patches. On day 33, two animals died after displaying muscular weakness, conjunctival and nasal secretions, and diarrhea. On day 35 the drug was discontinued in 11 mice. During the following days one mouse of this group died but the others recovered quickly and showed a marked reticulocytosis indicating the delivery of many young cells by the bone marrow was no longer inhibited by the drug. In 12 animals the drug was continued, 2 more died. Not all animals were usable, however, since the total amount of red blood cells was sometimes too small to allow the assays.

Enzyme changes. Enzyme activities and reticulocyte counts during and after the period of actinomycin D treatment are shown in Fig. 1 along with values obtained in 19 normal animals. Enzyme assays were performed in duplicate; these usually agreed within less than 5%. During the whole period of arrest of erythropoiesis, the activities of G-6-PD and 6-PGD were not clearly different from the normal range. Activity of GOT, however, seemed to decline during the first days, and thereafter stabilized a new, lower, level. The sudden influx of very young cells, occurring after discontinuation of the drug, was reflected by marked increases in G-6-PD and GOT. When, at the end of the recovery period, the reticulocytes had declined to the normal range, these enzymes were still elevated. Activity of 6-PGD showed only slight elevation during the period of reticulocytosis.

In order to obtain information of the density of the red cells during our experiment we used the density distribution technique of Danon and Marikovsky (5). By this method the behavior of red cells in a series of nonaqueous environments, differing in density, is recorded as an easily-read density profile. Curves were made on days 26, 28, and 33 of drug treatment (Fig. 2A) and on day 12 and 14 of the recovery phase (Fig. 2B). These results indicate that the mouse erythrocytes become denser as they age and that the new cells are much lighter. Technical difficulties including very small blood samples made it impossible to get complete data at the exact

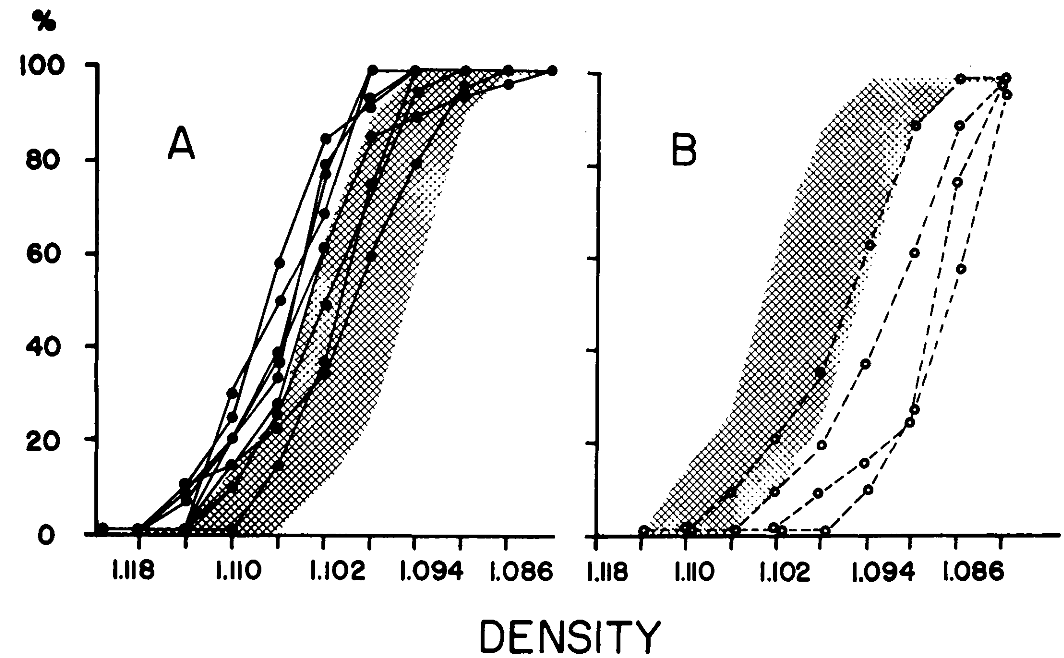

FIG. 2. Density distribution of red cells after prolonged administration of actinomycin D (A) and after cessation of drug administration (B). The shaded areas encompass the values of 6 normal mice. Each point represents the percentage of cells heavier than the density given.

transition point from suppression to release of erythropoiesis.

Discussion. As demonstrated earlier by Reismann and Ito (4), actinomycin D proved to be a useful tool in arresting the erythropoiesis in mice over a long period. These authors administered the drug over a period of 3 weeks. We were able to extend this period to 5 weeks and in some mice even longer, but then the toxicity of the drug and the anemia became a serious problem. Comparison of such a period with the life span of mouse red blood cells, recorded in the literature as about 40–55 days (8), makes it clear that the cells that remain must be close to the end of their life span. It is thus likely that any change occurring concomitant with cell aging would be detectable. In addition we were able to investigate the very young cell population appearing in the recovery phase.

The inhibitive action of actinomycin D on dividing tissues is thought to be due to selective suppression of DNA-dependent RNA synthesis (9). This cytostatic drug is highly toxic for the human (10), dog, and rabbit (11), but mice and rats appear to be less sensitive (11). Besides the erythropoietic tissue, many other tissues are also affected, as was apparent in the general symptoms in our mice. Both the general effects and the toxicity are limitations of this system for studying red cell aging.

Except for GOT, our results do not show any clearcut decline in enzyme activity during the period of actinomycin D treatment (Fig. 1). The decline in activity of GOT was restricted to the first days. After that it remained fairly constant. Our findings, therefore, disagree with the commonly held view that enzymes decline in activity with cell aging. This view is based to a great extent on studies with centrifugal fractionation. In these studies red blood cells from the bottom layer, often designated as "old" cells, are compared to cells from the top layer, "young" cells, and the observed differences in enzyme activity are ascribed to cell aging. The top layer, however, is likely to contain a significant percentage of reticulocytes. This means that, in fact, a mixed cell population is compared and since reticulocytes are metabolically much more active, it is not surprising that enzyme activities in the top layer are much higher. The decline of GOT during the first days of the present experiment parallels roughly the disappearance of reticulocytes. This effect could be demonstrated only for GOT, probably since this enzyme has a very high activity in reticulocytes as was also apparent from the results obtained in the recovery period. Sass and Spear (12) and Vuopio (13) demonstrated high red blood cell G-6-PD and GOT activity in anemias characterized by high levels of reticulocytes. The present data obtained during the recovery phase, which is also characterized by high levels of reticulocytes, are in agreement with their observations. No distinctive changes, however, could be noted in the activity of 6-PGD. The increased activities of G-6-PD and GOT were still present even when the reticulocytosis had almost disappeared. The same phenomenon was observed for GOT by Sass and Spear (12) in their patients recovering from anemia. One explanation for this phenomenon is some persistence of elevated enzyme level after reticulocyte maturation. Another explanation is that, after the long sustained arrest of erythropoiesis, the bone marrow at first delivers macrocytic cells with larger amounts of enzymes. Such macrocytosis following recovery from anemia has been described in rats (14), rabbits (15), and also in mice (16).

It is difficult to reconcile our data with that of Löhr and Waller (17), obtained in man, by differential agglutination (Ashby technique). In a fashion similar to the actinomycin D method, the aging effects could be studied by taking samples at successive intervals. Contrary to our results, Löhr and Waller found that as the red cells aged, there was gradual decline in activity of G-6-PD and some other glycolytic enzymes. Perhaps the difference reflects differences between mouse and man. The substantial random red cell lysis in mice, resulting in a much shorter finite life span, may obscure the very end of the life period. The decline observed by Löhr and Waller, however, was exponential rather than biphasic. It should be mentioned here that, in addition to species differences, there are differences in methods, too. As already indicated, actinomycin D is a very toxic drug, which might have affected our observations. Undoubtedly, the Ashby technique is more physiological, but suffers from the built-in disadvantage that the infused cells are not surviving in their own circulation. In addition, Löhr and Waller performed their studies in patients with chronic pulmonary tuberculosis rather than in normal individuals. Clinical medicine is of little help since an age-dependent decline of enzyme activity has been described in Negroes suffering from G-6-PD deficiency (18) but not in Sardinians with the disease (19).

Our experiments show, contrary to the commonly held view, no progressive decline of enzyme activity during cell aging. The main determining factor in activity of these red cell enzymes appeared to be the presence or absence of reticulocytes.

Summary. Red cell G-6-PD, 6-PGD, and GOT were studied in mice during and after prolonged arrest of erythropoiesis by means of repeated subcutaneous injections of actinomycin D. No clear downward trend in the activities of any of these enzymes in the progressively aging cells was found except for GOT, which initially declined as the reticulocytes disappeared and then stabilized at a new lower level. Discontinuation of the drug evoked a marked reticulocytosis accompanied by a sharp increase of G-6-PD and GOT activity. These data suggest that the major factor determining the red blood cell enzymes level is the presence or absence of reticulocytes.

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Absorption of Glucose, Galactose, and Xylose in the Dog* (32873)

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In a previous investigation, glucose reduced the intestinal absorption of galactose in the conscious dog but no effect of galactose on glucose absorption was demonstrated (1). Since only glucose was measured by a specific enzyme, the present study reinvestigates the effects of these sugars on one another. This study also tests the possible inhibitory effects between glucose and xylose in the dog since glucose reduced xylose absorption in the rat (2) and xylose reduced glucose uptake by hamster intestinal mucosa (3).

Methods. Solutions containing 10–150 m*M* of test sugar alone or with 10–75 m*M* of inhibitor sugar, plus NaCl to adjust the total osmolarity to about 325 milliosmols/liter, were perfused without recirculation through upper jejunal Thiry-Vella fistulas for 1 hour. [Glucose (Glucostat), galactose (Galactostat), xylose (4), and chloride (5), were measured in the input and the effluent solutions.] After perfusion, loops were flushed with 100 ml of H_2O and the residual unabsorbed solute was measured. Residual loop volume was estimated as the amount of flushed solute divided by the solute concentration in the effluent solution. Net absorption of fluid or solute was considered to be the amount infused minus the sum of effluent and loop residual amounts.

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Two series of experiments were done. In the first, 5 concentrations of glucose, 5 of galactose, and 7 combinations of glucose and galactose concentrations were presented in random order using random number tables, and the 17 test solutions were repeated according to a second randomization. Glucose or xylose alone, and 5 combinations of the two sugars were tested similarly in the second series. Five dogs were used for both series, each tested on alternate days after 18 hours' fasting.

Results. The experimental data are summarized in Table I. The means were calculated by averaging duplicate tests on each dog, then averaging the results obtained in all 5 dogs. The first two columns for each pair of sugars estimate the average concentration to which the loops were exposed for 1 hour; this was calculated as the average of input and effluent concentrations. Table I shows that the addition of glucose reduced galactose absorption rates whether concomitant net fluid absorption rates increased or decreased. In the other combinations, the addition of a second sugar had inconsistent effects on the absorption of the test sugar and usually altered net fluid absorption rates.

Since a previous similar study has shown that hexose absorption is correlated positively with the rate of net fluid absorption (6), the