Inactivation of Erythropoietin by Hepatic Lysosomes[1] (37599)

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The liver has been implicated as a primary site of inactivation of erythropoietin (erythropoietic stimulating factor, ESF) in several *in vivo* studies in rabbits and rats (1–6). Burke and Morse, (7), using an isolated hepatic perfusion system, have reported that normal livers could significantly reduce the erythropoietic activity of perfusates containing high titers of ESF. Livers from carbon tetrachloride-pretreated animals produced markedly less reduction in the erythropoietic activity of these perfusates than normal livers.

More recently, Roh, Paulo and Fisher (8) have studied the inactivation of ESF in perfusions of hind limbs and livers from control and drug-pretreated dogs. They evaluated the effects of the microsomal enzyme inducer, phenobarbital, as well as the acute effects of the micrsosomal enzyme inhibitor, SKF 525-A, on liver inactivation of ESF. Although they observed that the hepatic inactivation of ESF was significantly inhibited by SKF 525-A, this inactivation process was not affected by phenobarbital pretreatment. These investigators suggested that the inhibition of ESF inactivation following SKF 525-A pretreatment may be due to a different mechanism than microsomal enzyme inhibition.

While all these experiments indicate that the liver is involved in the inactivation of ESF, no information is provided concerning the intracellular site or mechanism of this inactivation.

The present investigation was undertaken to study the *in vitro* hepatic inactivation of ESF in order to clarify the mechanism of ESF inactivation, and to attempt to localize the actual subcellular site(s) of this inactivation.

Materials and Methods. Mongrel dogs (8–12 kg) of either sex and male Sprague–Dawley rats (200–250 g) served as liver donors in all studies. Mongrel dogs of the same size and male Sprague–Dawley rats greater than 300 g were used as blood donors. HAM/ICR strain female mice (22–25 g) were used in the assay of ESF.

The sodium salt of phenolphthalein glucuronic acid, glycine, and standard biochemical reagents were obtained from Sigma Chemical Co. All other reagents were purchased from Mallinckrodt Chemical Works.

Plasma from hypoxic dogs (HDP) or serum from hypoxic rats (HRS) containing high titers of ESF was used as the source of ESF in these studies. To obtain the plasma and serum, dogs or rats were made hypoxic by exposure to 0.42 atm pressure in a hypobaric chamber for 18 hr. Immediately after removal from the hypobaric chamber, the dogs were anesthetized with pentobarbital and exsanguinated via the carotid artery. The rats were exsanguinated via cardiac puncture while under ether anesthesia. The HDP and pooled HRS were assayed for erythropoietic activity in exhypoxic polycythemic mice according to a modification of the method of Cotes and Bangham (9). In this assay, mice were placed in a hypobaric chamber (0.42 atm) for 2 wk to induce polycythemia. After removal from the chamber the mice were injected subcutaneously with ½ the total dose of the assay sample on each of the fourth and fifth posthypoxic days. Twenty-four hours later, 0.5 μCi radiolabeled ferrous citrate (^{59}Fe) was injected via the tail vein. On the eighth posthypoxic day the mice were exsanguinated via cardiac puncture and the

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percentages of ^{59}Fe incorporations into red blood cells were determined. Previous studies have established that the erythropoietic activity of hypoxic blood is blocked by the antibody to ESF (10). This demonstrates that the erythropoietic activity induced by hypoxia is due to elevated levels of ESF, rather than to other blood constituents.

Dog livers were quickly excised and placed in cold buffer. All subsequent operations were performed in a cold room (0–4°). A Potter–Elvehjem tissue grinder was used to prepare liver homogenates (25% w/v) in 0.32 *M* sucrose containing 0.02 *M* Tris (pH 7.6) and 3 m*M* $MgCl_2$. The cell fractionation procedure of Mahler and Cordes (11) was followed to prepare particulate (24,000*g* pellet), microsomal (105,000*g* pellet), and soluble liver fractions. Incubations of HDP with each of the liver fractions were conducted in a medium containing Sorensen phosphate buffer (pH 7.4), 10 units glucose-6-phosphate dehydrogenase, 1 m*M* glucose-6-phosphate, 0.15 m*M* NADP, 2 m*M* $MgCl_2$, and 1.15% KCl. These incubation mixtures, which also included 4 IRP units of ESF from HDP (1 unit/ml) and subcellular liver fractions equivalent to 2.8 g of dog liver, were carried out for 1 hr at 37° in a Dubnoff metabolic shaker. Following incubation the samples were frozen at —20° until assayed for ESF.

Rat liver fractions were obtained from the pooled livers of 4 or more animals. All animals were fasted 24 hr and sacrificed by decapitation. The livers were immediately excised, cut into small pieces, and placed in cold 0.25 *M* sucrose buffer. All subsequent operations were performed in the cold room. The tissues were homogenized in a Waring blender at medium speed for 20 sec. The resulting homogenates were diluted in 0.25 *M* sucrose such that each milliliter of the resulting preparation contained from 0.25–0.50 g of wet weight tissue. These homogenates were then centrifuged in a Sorvall RC 2-B centrifuge at 1500*g* for 15 min and the resulting supernatant fraction was centrifuged at 19,600*g* for 20 min. The original homogenate and all other fractions were assayed for β-glucuronidase activity (12) and incubated with HRS for 6 hr at 37° in a Dubnoff metabolic shaker. Incubations included 2 IRP units ESF from HRS (1 unit/ml) and subcellular fractions equivalent to 0.8–4.0 g of rat liver. The pH in these incubation mixtures was approximately 7.0 and no additional buffers were employed. Following incubation the flasks were quickly frozen at —20° until assayed for ESF.

Lysosomes were prepared by the method of Sawant *et al.* (13). Various amounts of this fraction were incubated with HRS for 0 to 6 hr as described above, but with the pH lowered to 4.2 by addition of sodium acetate buffer. In certain experiments the lysosomal fractions were heated for 7 min in a boiling water bath to denature the lysosomal proteins prior to incubation with HRS.

Results. Figure 1 shows the effect of dog liver subcellular fractions on the erythropoietic activity of HDP. Significant inactivation ($p < 0.05$) of ESF occurred after incubation with the particulate liver fraction, but not after incubation with other subcellular fractions. When incubated alone, none of the liver fractions contained significant erythropoietic activity. These results suggest that a component of the particulate fraction such as the lysosomes is responsible for inactivating ESF.

To determine whether the lysosomes were involved in ESF inactivation, rat liver subcellular fractions were incubated with HRS. The degree of ESF inactivation exhibited by these fractions was well correlated with the activity of the lysosomal marker, β-glucuronidase. Figure 2 illustrates a correlation between the β-glucuronidase activities and the loss of erythropoietic activity in HRS. The high correlation coefficient of —0.98 strongly suggests that the lysosomes are involved in the inactivation of ESF.

To further evaluate the ability of hepatic lysosomes to inactivate ESF, lysosomal fractions were prepared (13) and incubated with HRS. These incubations were carried out at a pH of 4.2 because most lysosomal enzymes are known to have pH optima in the acid range. This low pH should also facilitate the labilization of the lysosomal particles, thus insuring the release of lysosomal

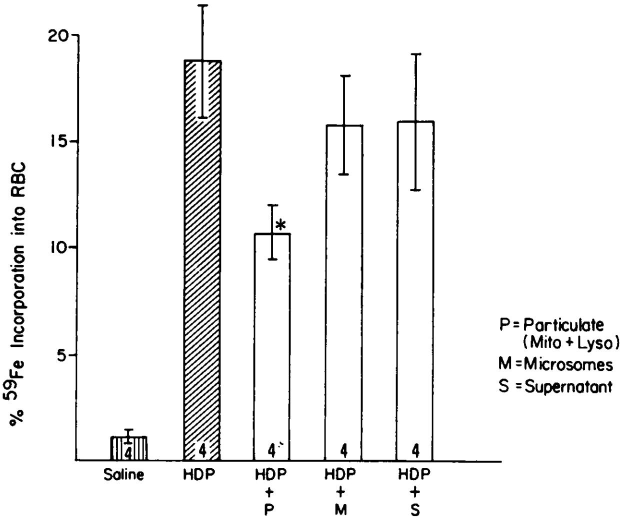

FIG. 1. Effect of dog hepatic subcellular fractions on the erythropoietic activity of hypoxic dog plasma (HDP). Each bar represents the mean of 4 experiments ± standard error. Each experiment utilized 5 mice per assay group. Saline bar represents mice injected with normal saline solution and serves as a control to measure the endogenous erythropoiesis in the assay mice. Asterisk denotes significant difference ($p < 0.05$) from HDP incubated alone. All incubations were for 1 hr at 37° and pH 7.4.

enzymes to act on the HRS substrate. Figure 3 illustrates the results of incubating HRS with rat hepatic lysosomal fractions. Significant inactivation is detectable after 15 min

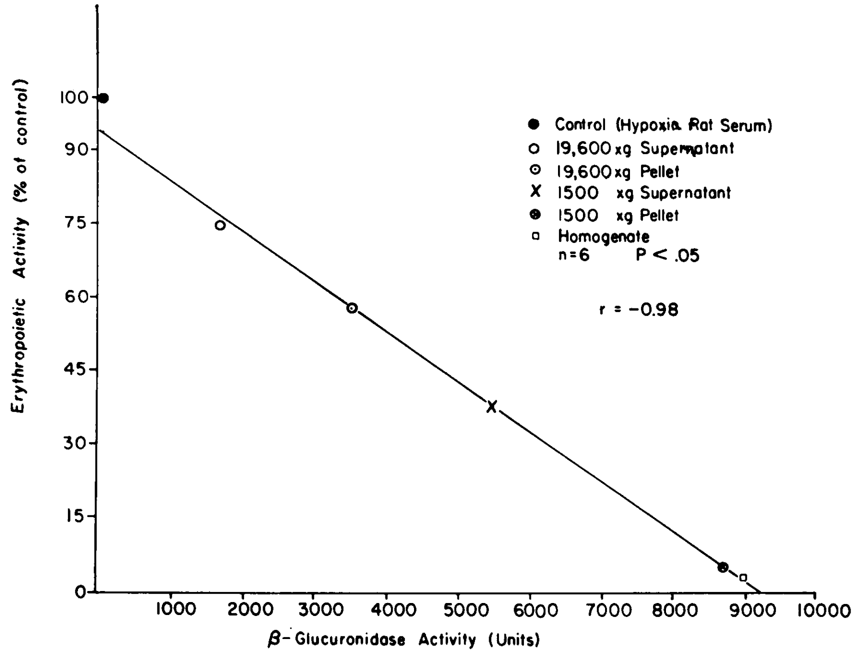

FIG. 2. Correlation between activity of the lysosomal marker β-glucuronidase and degree of inactivation of erythropoietin in hypoxic rat serum. Correlation coefficient (r) equals —0.98. One unit β-glucuronidase activity liberated 3 nmoles phenolphthalein under the conditions of the assay. Incubations were for 6 hr at 37° and pH 7.0.

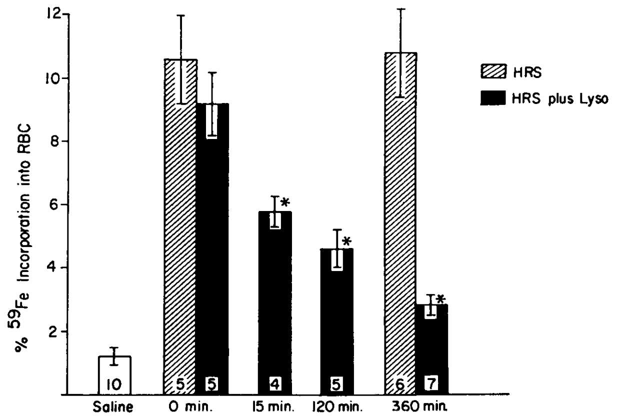

FIG. 3. Inactivation of erythropoietin in hypoxic rat serum (HRS) by rat liver lysosomes. Numbers within bars denote number of experiments. Each experiment utilized 5 mice per assay group. Asterisks denote significant difference ($p < 0.05$) from *HRS plus lysosomes* at zero time of incubation and also from *HRS alone* at zero time and after 6 hr incubation. All incubations were at 37° and pH 4.2.

of incubation. After 6 hr the erythropoietic activity was reduced to less than 25% of the original level. HRS incubated at pH 4.2 without the lysosomal fraction retained all of its activity throughout the 6 hr incubation.

Figure 4 depicts data from experiments in which the concentration of lysosomes in the incubation flask was varied from 12 to 50%. The amount of ESF inactivation was greater with increased lysosomal content, as would be expected in cases of enzymatic inactivation.

If the lysosomal inactivation of ESF is indeed enzymatic, heating the liver fraction prior to incubation should denature the enzymes involved and reduce the degree of ESF inactivation. As seen in Fig. 5, heating the lysosomes for 7 min in a boiling water bath destroyed the ability of the lysosomal fraction to inactivate ESF.

Discussion. In the present studies, dogs and rats were used to localize the hepatic subcellular site of erythropoietin inactivation. Our data clearly indicate that the inactivation mechanism is associated with the lysosomal fraction. The role of the lysosomes in inactivating ESF is supported by the use of various fractionation techniques and different incubation conditions. Furthermore, the enzyme activity associated with this lysosomal fraction either possesses a broad pH optimum or else is a combination of two or more ESF-inactivating enzymes.

It is not possible to completely rule out ESF inactivation by enzymes from other than lysosomal sources because nonlysosomal liver subcellular fractions produced marginal in-

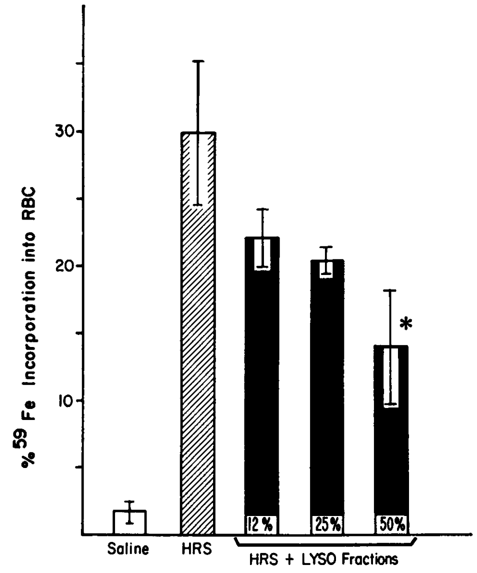

FIG. 4. Effect of varying rat liver lysosomal protein concentrations on the inactivation of erythropoietin in hypoxic rat serum. Bars represent the mean ± SEM of 5 mice per assay group. Asterisk denotes significant difference ($p < 0.05$) from HRS incubated alone. All incubations were for 6 hr at 37° and pH 4.2.

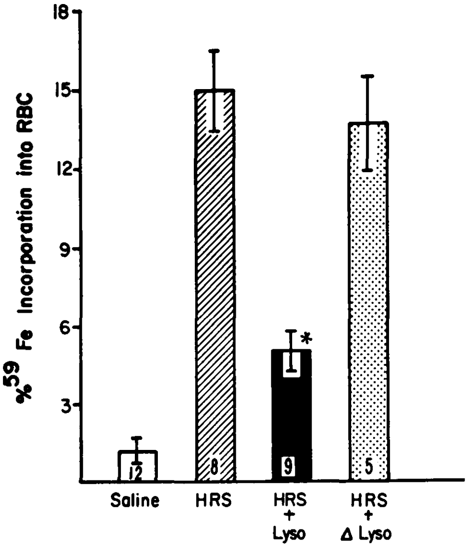

FIG. 5. Effect of heat denaturation on the ability of lysosomes to metabolize erythropoietin. Numbers within bars denote number of experiments. Each experiment utilized 5 mice per assay group. Asterisk denotes significant difference ($p < 0.05$) from HRS incubated alone. Heat denaturation of lysosomes (Δ Lyso) prior to incubation was for 7 min in a boiling water bath. Incubations were for 6 hr at 37° and pH 4.2.

activation of ESF. However, all ESF inactivation was well correlated with the activity of β-glucuronidase, an enzyme used as a lysosomal marker. Therefore, the activity observed in these other fractions was probably due to lysosomal contamination. Clearly, the present results provide good evidence that the highest ESF inactivation *in vitro* occurs in hepatic lysosomes and that this inactivation is enzymatic.

It is not clear whether our *in vitro* studies on hepatic lysosomal inactivation are related to the physiological mechanism for erythropoietin metabolism. However, ESF is a plasma glycoprotein similar to other plasma glycoproteins such as fetuin and orosomucoid, which are extensively degraded by hepatic lysosomes, as was reported by Aronson and DeDuve in 1968 (14). These observations have been extended by other investigators who have shown that certain macromolecular compounds are taken up, concentrated and destroyed by lysosomes (15–18). Gregoriadis *et al.* (19) reported that desialylated ceruloplasmin was rapidly cleared by the liver and transported to the lysosomes where it was metabolized. These studies therefore indicate that hepatic lysosomes are indeed involved in the physiological inactivation of many plasma glycoproteins. Thus, these earlier reports further support our findings in the present investigation which suggest that the metabolism of ESF by the hepatic lysosomes is a physiological process by which plasma titers of ESF may be regulated.

Summary. Studies were performed to determine the effects of liver homogenates and subcellular fractions on the inactivation of ESF. ESF was assayed using the percentage of ^{59}Fe incorporation into the erythrocytes of exhypoxic polycythemic mice. Liver inactivation of ESF was found to correlate with the activity of β-glucuronidase, a lysosomal marker enzyme. Incubation of ESF with hepatic lysosomal fractions resulted in a reduction in erythropoietic activity which was significant after 15 min. Further progressive inactivation occurred over a period of 6 hr. Decreasing the content of the lysosomal protein in the reaction flasks or heat denaturation of the lysosomal fraction substantially reduced the amount of ESF inactivation, providing additional evidence that this process is enzymatic.

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1. Jacobsen, E. M., Davis, A. K., and Alpen, E. L., Blood **11**, 937 (1956).
2. Prentice, T. C., and Mirand, E. A., Proc. Soc. Exp. Biol. Med. **95**, 231 (1957).
3. Prentice, T. C., and Mirand, E. A., Exp. Med. Surg. **15**, 176 (1957).
4. Mirand, E. A., Prentice, T. C., and Slaunwhite, W. R., Ann. N.Y. Acad. Sci. **77**, 677 (1959).
5. Alpen, E. L., *in* "Erythropoiesis" (L. O. Jacobson and M. Doyle, eds.), p. 134. Grune & Stratton, New York (1962).
6. Murphy, G. P., Mirand, E. A., Steeves, R. A., and Melby, E. C., Invest. Urol. **7**, 283 (1970).
7. Burke, W. T., and Morse, B. S., *in* "Erythropoiesis" (L. O. Jacobson and M. Doyle, eds.), p. 111. Grune & Stratton, New York (1962).
8. Roh, B. L., Paulo, L. G., and Fisher, J. W., Amer. J. Physiol. **223**, 1345 (1972).

9. Cotes, P. M., and Bangham, D. R., Nature (London) **191**, 1065 (1961).

10. Fried, W., Kilbridge, T., Krantz, S., McDonald, T. P., and Lange, R. D., J. Lab. Clin. Med. **73**, 244 (1969).

11. Mahler, H. R., and Cordes, E. H., "Biological Chemistry," 2nd ed., p. 448. Harper & Row, New York (1971).

12. Ignarro, L. J., Biochem. Pharmacol. **20**, 2847 (1971).

13. Sawant, P. L., Shibko, S., Kumta, U. S., and Tappel, A. L., Biochim. Biophys. Acta **85**, 82 (1964).

14. Aronson, N. N., Jr., and DeDuve, C., J. Biol. Chem. **243**, 4564 (1968).

15. Dingle, J. T., and Barrett, A. J., Proc. Roy. Soc. Ser. B. **173**, 85 (1969).

16. Gabathuler, M. P., and Ryser, H. J. P., Proc. Roy. Soc. Ser. B. **173**, 95 (1969).

17. Mahadevan, S., Nduaguba, J. C., and Tappel, A. L., J. Biol. Chem. **242**, 4409 (1967).

18. Tulsianai, D. R. P., and Carubelli, R., J. Biol. Chem. **245**, 1821 (1970).

19. Gregoriadis, G., Morell, A. G., Sternlieb, I., and Scheinberg, I. H., J. Biol. Chem. **245**, 5833 (1970).

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