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Multiple Forms of Biliverdin Reductase: Age-Related Change in Pattern of Expression in Rat Liver and Brain

MAHIN D. MAINES
University of Rochester School of Medicine, Department of Biophysics, Rochester, New York 14642
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SUMMARY

Biliverdin reductase is the dual nucleotide-dependent cytosolic enzyme that converts biliverdin to the bile pigment, bilirubin, and displays extensive microheterogeneity in rat organs. The enzyme is unique in having two pH optima. The present study reports on the tissue-dependent pattern of developmental expression of the reductase in rat liver and brain. When analyzed by Western immunoblotting, two closely migrating immunoreactive proteins were detected in the liver cytosol during the first 2–3 weeks after birth; the protein with greater mobility was not detected in the liver of adult aged animals (6 months old) and was present at low levels in rats during the first week of life. The faster migrating protein was not detected in the brain cytosol at any stage of development. Furthermore, in the brain the total amount of enzyme protein increased as the animal matured, whereas in the liver the enzyme protein level decreased with age. When the purified enzyme was analyzed, age-related changes in the variant composition of the enzyme in the liver were noted. Although in both adult and newborn animals (14 days old) the purified enzyme, when subjected to isoelectric focusing, separates into five net charge forms (pI 6.23, 5.91, 5.76, 5.61, and 5.48), the relative abundance of the variants notably differed in the two preparations. In addition, when the purified preparations were subjected to two-dimensional electrophoresis, although both purified preparations separate into three molecular weight forms (M_r 30,400, 30,700, and 31,400) one species (M_r 31,400, pI = 5.77), which was very prominently expressed in the newborn, was essentially absent in the adult. Biliverdin reductase activity of the liver cytosol with both NADPH (pH 8.7) and NADH (pH 6.7) exhibited developmental changes, with the activity increasing after birth, reaching a peak on day 14, and decreasing to low levels in the adult. The existence of a close correlation between development of biliverdin reductase activity in the brain and liver and that of heme oxygenase in these organs is noted. The suggestion is made that the reductase is not a passive component of the heme degradation pathway; rather, its activity could become limiting in the elimination of heme degradation products.

The terminal stage of heme *b* degradation in mammalian species, i.e., the conversion of biliverdin IXα to bilirubin IXα, is catalyzed by the cytosolic enzyme biliverdin reductase (1–3). This reaction exploits the reducing potential of pyridine nucleotide coenzymes for the reduction of the γ-methene bridge of biliverdin. Studies with purified preparations of the enzyme have shown that, although the reduction of biliverdin can be supported by either NADH or NADPH, each cofactor has a different pH optimum (3, 4). Although a few other enzymes are known that can utilize two pyridine nucleotide coenzymes, biliverdin reductase is unique among all enzymes in higher animals with respect to having two distinct pH optima.

Recently, we have detected an extensive microheterogeneity of the reductase in the rat liver, spleen, and kidney (5–8). Using two-dimensional electrophoresis, the purified control liver enzyme resolved into five net charge groups, with pI values of 6.23, 5.91, 5.76, 5.61, and 5.48, and three molecular weight groups, with M_r 30,400, 30,700, and 31,400 (5). When selected net charge groups were examined, differences were noted in their catalytic activities, amino acid compositions, and peptide maps (5, 6).

Biliverdin reductase is not generally considered rate limiting in heme degradation; rather, microsomal heme oxygenase is believed to serve in this capacity. Recently, two forms of heme oxygenase, HO-1 and HO-2, have been identified (9–11), with marked differences in their developmental pattern of gene expression in the brain (12) and liver.[1] The high level of HO-1 in the livers of newborn rats results in a high level of heme degradation activity by this organ during the first 2 weeks of postnatal development (13, 14). In contrast, brain heme oxygenase activity and mRNA for the isozymes in the newborn rat are present at low levels during the first 2 weeks of life and are increased as the animal reaches maturity (12). Because the product of heme oxygenase activity, biliverdin, serves as the substrate for the reductase, we reasoned that the activity of

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[1] Unpublished observations.

ABBREVIATIONS: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

biliverdin reductase also may undergo developmental regulation. The present study was undertaken to examine this hypothesis and to discern whether there are age-related changes in the tissue levels of different variants of the reductase.

Experimental Procedures

Materials. Most chromatographic materials, ampholytes, and various biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). NADP-agarose (type 3), Polybuffer exchanger 94, Polybuffer 96, and Polybuffer 74 were purchased from Pharmacia-LKB (Piscataway, NJ), and Ultrogel AcA54 was purchased from IBF Biotechnics (Columbia, MD). Biliverdin IXα was obtained from Porphyrin Products (Logan, UT). All chemicals were of the highest purity commercially available. Goat anti-rabbit IgG conjugated with alkaline phosphatase was purchased from Organon Teknika Corp. (West Chester, PA). Nitrocellulose filters with a 0.2-μm pore size were from Schleicher and Schuell. Pregnant female Sprague-Dawley rats were purchased from Harlan Industries (Madison, WI), and male New Zealand rabbits (3–4 kg) were purchased from Hazleton Research Animals (Denver, PA). The liver cytosol fraction was prepared from the livers of rats at the following stages of development: −1, 1, 4, 7, 21, or 39 days, or ≈6 months, as described previously (5).

Enzyme purification. Biliverdin reductase from 14-day-old or adult rat liver was purified to homogeneity by the method previously described (1), but with the following modifications. Livers were homogenized in 4 volumes of a 20.0 mM potassium phosphate buffer, pH 7.4, that contained 1.0 mM EDTA and 135.0 mM KCl. The cytosol fraction, which was prepared by centrifugation at 150,000 × *g* for 1 hr, was diluted with 1/19 volume of a 1.0 M citrate buffer, pH 5.4, which contained 2.0 mM dithiothreitol, before ammonium sulfate fractionation. Group-specific chromatography with a NADP-agarose column was performed as detailed previously (3), except that the enzyme was eluted from the column with 50.0 mM glycine that was prepared in the equilibration buffer and adjusted to pH 9.5. The active fractions were pooled, concentrated using an Amicon YM10 Diaflo membrane, and subjected to gel filtration using an Ultrogel AcA54 chromatographic column; the mobile phase contained 0.1 mM dithiothreitol. The active fractions were pooled and concentrated.

Electrophoretic and immunochemical methods. SDS-PAGE was performed by the method of Laemmli (15). The separating gels contained 10.0% *T* (i.e., total acrylamide concentration, w/v) and 2.67% *C* (i.e., the ratio of bisacrylamide to total acrylamide, w/w), and the stacking gels contained 3.0% *T* and 2.67% *C*. β-Galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,400), serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 29,000), and trypsinogen (M_r 24,000) were used as molecular weight markers.

The method used for polyacrylamide gel IEF was based upon that previously described by Righetti and Drysdale (16). The separating gels contained 4.0% *T* and 5.4% *C*. Initially, the pH 5–8 range and pH 3.5–10 range carrier ampholytes were combined to examine the full range of biliverdin reductase pI values. In this case, ampholyte concentrations were adjusted to 1.8% (w/v) and 0.2% (w/v), respectively. Subsequently, ampholytes with a pH 4–6.5 range were used instead of a pH 5–8 range. Electrofocusing was performed at 400 V for 17 hr and then continued for 1.5 hr at 800 V. Amyloglucosidase from *Aspergillus oryzae* (pI 3.55), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.13), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.57), and horse heart myoglobin (pI 6.76 and 7.16) were used as pI markers.

Two-dimensional PAGE was performed essentially by the procedure of O'Farrell (17). The conditions that were used for both the first-dimension IEF tube gel separation and the second-dimension SDS-PAGE separation were similar to those that have been described above for the single-dimension separations.

The method of Righetti and Drysdale (16) was used when IEF gels were stained with Coomassie brilliant blue R250. The positive-image silver staining method of Wray *et al.* (18) was used for the staining of two-dimensional PAGE gels. Laser densitometry and automatic integration were performed with an LKB Ultroscan XL densitometer.

Antibody to rat liver biliverdin reductase was prepared in New Zealand rabbits by the method previously described (5). Primary and secondary antibody treatments of the Western immunoblots were performed as described previously (12).

Assay procedures and calculations. Protein was measured by the method of Lowry *et al.* (19). Bovine serum albumin was used as the protein standard. Routine measurements of biliverdin reductase activity were performed as described previously (5). The assay method previously detailed (3, 5) was used to determine the pH-dependent cofactor requirements of biliverdin reductase. The reaction was monitored for 30 to 60 sec at 25°. An extinction coefficient of 53 $mM^{-1}cm^{-1}$ at 450 nm was used (3).

Results

The developmental pattern of rat liver biliverdin reductase, as visualized by Western immunoblotting, is shown in Fig. 1. For this experiment, equal amounts of cytosol protein (300 μg) obtained from the pooled livers of three to five rats were loaded in each well; biliverdin reductase purified from the livers of adult rats was used as the standard. As shown, a protein with immunoreactivity and SDS gel migration behavior similar to that of the purified biliverdin reductase was present in the livers of rats at all stages of development and maturation including −1, 1, 4, 7, 14, 21, and 39 days, as well as in old adult animals. The intensity of the band for the adult animal was lower than that observed at other stages of development. In addition, a second immunoreactive protein was detected in samples obtained from rats at all stages of development except for old adults. The intensity of this band, however, differed in the samples, with the band being the most prominent in cytosol samples obtained from the livers of 7-, 14-, and 21-day-old rats.

The tissue profile of biliverdin reductase in developing rats was further examined by comparing that of the liver and the brain. Brain cytosol from rats at −1, 1, and 14 days after birth and old adult animals was subjected to Western immunoblotting. For proper comparison, the liver cytosol from the same animals was also examined. The results are shown in Fig. 2. A distinctly different pattern of development of the enzyme was detected in the brain. In this organ (Fig. 2A), the tissue level of the enzyme assessed by laser densitometry of bands visualized in immunoblots, which was low in the −1- and 1-day-old animals, was increased by 2-fold in 14-day-old animals and again by 2-fold in old adult rats. Furthermore, the second immunoreactive protein was not detected in the brain samples at any stage of development. When electrophoresed in adjacent

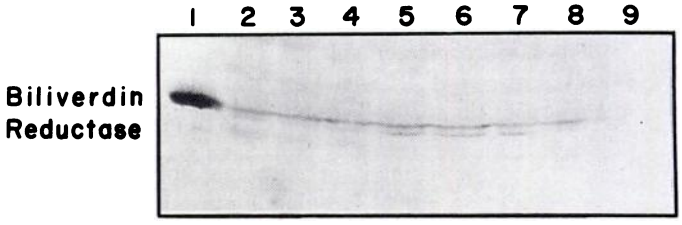

Fig. 1. Western immunoblot of biliverdin reductase in rat liver cytosol. The liver cytosol fraction was prepared from rats at different stages of development and subjected to SDS-gel electrophoresis, followed by electroblotting onto a nitrocellulose sheet. Subsequently, the blot was immunochemically stained, as described in Experimental Procedures. *Lanes 1–9* (in order): purified liver biliverdin reductase, cytosol from liver of −1-, 1-, 4-, 7-, 14-, 21-, or 39-day-old rats, or cytosol from adult (≈6-month-old) rats. The amount of protein in cytosol samples was 300 μg.

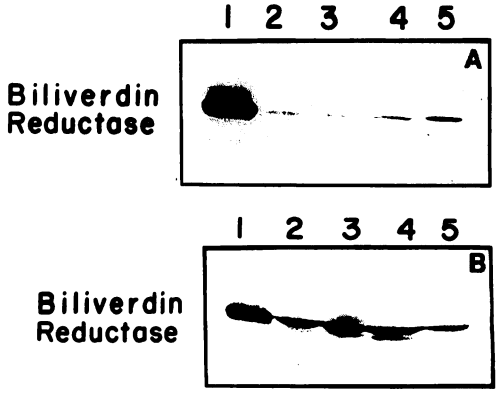

Fig. 2. Comparative development of rat liver and brain biliverdin reductase. The cytosol fraction was prepared from the brain or the liver of rats at different stages of development. The preparations were subjected to SDS-gel electrophoresis, followed by electroblotting onto a nitrocellulose sheet. The blot was immunochemically stained as detailed in the text. *Lanes 1–5* (in order): purified liver biliverdin reductase, 300 μg of cytosol protein from tissues of −1-day-, 1-day-, or 14-day-old or old adult rats. A, Brain; B, liver.

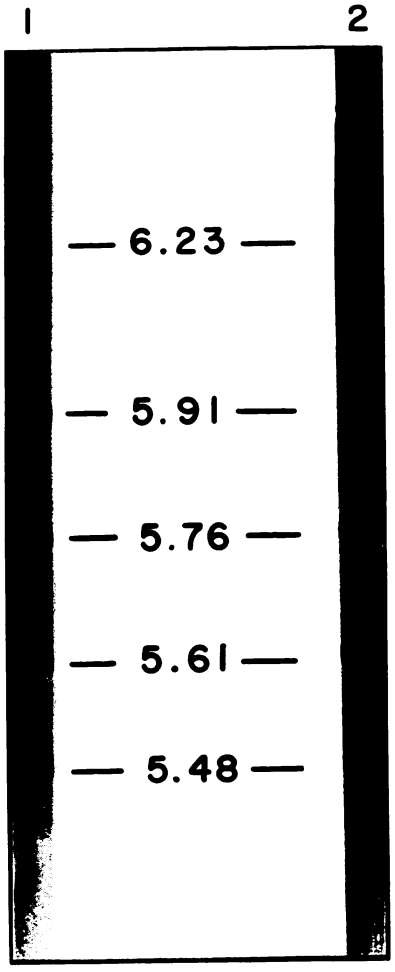

Fig. 3. Tube gel IEF of old adult and 14-day-old rat liver biliverdin reductase. Biliverdin reductase was purified from the liver of old adult male Sprague-Dawley or 14-day-old rats. Six micrograms of purified enzyme preparation were separated by IEF, as described in Experimental Procedures.

TABLE 1

Quantitation of the relative amount of biliverdin reductase net charge variants in the liver of 14-day-old and old adult rats

Calculation was based on densitometric quantitation of replicate IEF separations of purified biliverdin reductase preparations, examples of which are shown in Fig. 3. In all cases, variation between the replicates did not exceed 10% of the indicated mean values.

IEF	pI	Relative amount of variants	
		Aged	14-day-old
		%	
1	6.23	12.4	10.6
2	5.91	17.1	8.6
3	5.76	22.5	38.2
4	5.61	38.7	35.7
5	5.48	9.3	6.9

channels on the same gel, followed by immunoblotting, the single immunoreactive band of the brain corresponded to the major band of the liver (data not shown). The results obtained with the liver (Fig. 2B) were consistent with those obtained in the previous experiment (Fig. 1).

The developmental aspects of heterogeneity of liver biliverdin reductase were examined (Fig. 3). Biliverdin reductase was purified from the liver of old adult (Fig. 3, *lane 1*) and 14-day-old (Fig. 3, *lane 2*) rats and subjected to tube gel IEF. Both tubes, which were run in duplicate, contained the same amount of protein (6 μg) and the protein bands were visualized by Coomassie blue. Both preparations resolved in five electrophoretic zones, with pI values of 6.23, 5.91, 5.76, 5.61, and 5.48, and were designated as IEF-1 to -5, respectively. The relative composition of each IEF fraction, as determined densitometrically, is shown in Table 1. As noted, major differences were observed in relative intensities of the variants. For instance, in the 14-day-old animals the relative intensity of IEF-3 was about 70% higher than that in the adult animal and that of IEF-2 was only 50% of that in the adult. The relative values for IEF-1 and-5 were also lower in 14-day-old rats, with that of IEF-4 being rather similar in both preparations.

In order to ascertain whether the molecular weight composition of the various biliverdin reductase net charge variants differs in the two age groups, the purified preparations were subjected to two-dimensional PAGE (Fig. 4). The first-dimension IEF tube gel was inoculated with 3 μg of protein. The reductase separated once again into five major net charge groups. As mentioned earlier, we have shown in the past (5) that, when the charge variants are subjected to SDS gel electrophoresis, they resolve into three molecular weight groups of about 30,400, 30,700, 31,400. A similar observation was also made in the present study. However, a striking difference between the two enzyme preparations was noted in the molecular weight species present in the IEF-3 charge variant (pI 5.76). In this charge variant, the molecular weight species of M_r 31,400 was prominently present in enzyme purified from the 14-day-old rats (Fig. 4A), whereas in the aged animals it was present in a minute amount (Fig. 4B).

In an attempt to relate the changes noted in the composition of biliverdin reductase to the biological activity of the enzyme, the activity of the reductase in the liver cytosol at different stages of development was measured. Results are shown in Fig. 5. As noted, the activity with both cofactors (NADPH at pH 8.7 or NADH at pH 6.7) gradually increased after birth and the peak activity was reached on day 14. Thereafter, it proceeded

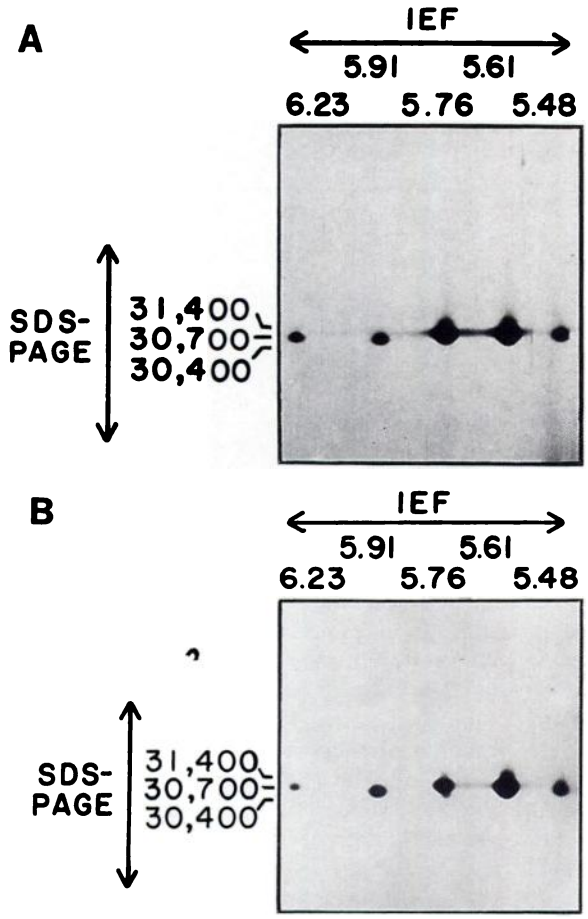

Fig. 4. Two-dimensional electrophoresis of purified adult and 14-day-old rat liver biliverdin reductase. In the first dimension, 3 μg of purified biliverdin reductase protein were separated by tube gel IEF. Subsequently, SDS-PAGE was used for the second dimension. Experimental details are provided in the text. A, 14-day-old; B, old adult.

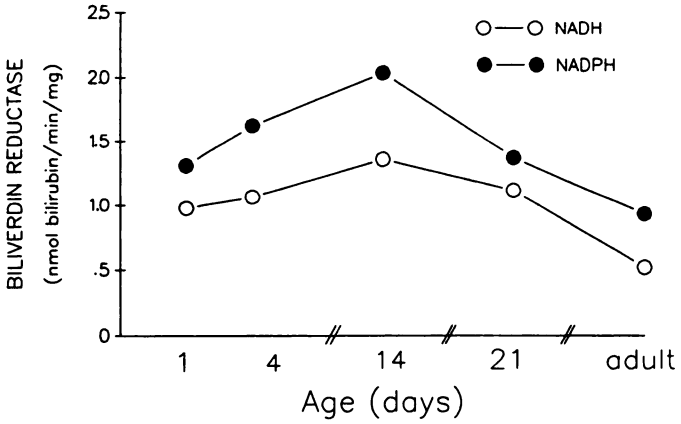

Fig. 5. Development of rat liver biliverdin reductase. The development of pH-dependent activity of rat liver biliverdin reductase at the indicated stages of development was measured at pH 6.7 and 8.7. The concentrations of NADH (at pH 6.7) and NADPH (at pH 8.7) in the reaction mixtures were 1.0 and 0.1 mM, respectively. These concentrations were found to be optimum for enzyme activity. The cytosol fraction was used as the enzyme source.

to decrease and reached the nadir in the adult animals. It is noteworthy that the pattern of development of activity was similar for both cofactors, although at later stages of development NADPH was a more effective cofactor for the enzyme.

Discussion

The present study reports on the developmentally regulated changes in the activity and molecular composition of biliverdin reductase in rat liver cytosol. The observed changes in the composition of molecular weight variants of the reductase are fully unexpected, and at this time the molecular basis for modulations is not clear. A plausible explanation for changes in the composition of the molecular weight variants, however, could reside in the possibility that variants reflect post translational modifications of the enzyme and that protein-processing events in the newborn vary from those of the older animals. The possibility that the molecular weight variants are different gene products and their expression is developmentally regulated is also a viable alternative. It is noteworthy that developmentally controlled modulation occurs in the composition of molecular weight variants of the enzyme but not in the net charge variants of the reductase.

The change in the composition of biliverdin reductase molecular weight variants in the course of development could be related to the developmental change in the activity of the enzyme (Fig. 5). The observed concerted changes in the variant composition and activity of the enzyme in the liver may be interpreted as suggesting that newborn animals possess a larger proportion of biliverdin reductase variants that are kinetically more active than other variants. The results of Figs. 1 and 5 indicate that, although the specific content of biliverdin reductase protein remains relatively constant, the specific activity of the enzyme is altered in an age-dependent manner. This suggestion is consistent with our previous observation, with a limited number of variants of biliverdin reductase, that net charge variants display different rates of catalytic activity (5, 6). The composition of biliverdin reductase variants is not only subject to developmental regulation, which is presently demonstrated, but as previously shown (7, 8) is also affected by exposure to chemicals. For instance, bromobenzene treatment of rats causes pronounced alteration in the variant composition of biliverdin reductase in the liver and the kidney (7, 8).

The observed difference in the pattern of development of the enzyme in the liver and the brain is rather intriguing, particularly because the developmental pattern of biliverdin reductase activity in the liver and the brain closely follows that of heme oxygenase in these organs (12–14). In the rat brain, which contains predominantly the HO-2 species (5, 20), exceedingly low levels of HO-2 mRNA and heme degradation activity are detected during the first 2 weeks of life (12). In contrast, in the rat liver the activity of heme oxygenase is elevated during the first 1–2 weeks of the postnatal period and gradually decreases to reach the adult value (13, 14). The rather intriguing correlation that emerges when the developmental patterns of heme oxygenase and biliverdin reductase are examined may well signify the biological importance of changes in the composition of biliverdin reductase and its direct role in enabling the animal to dispose of heme degradation products. Because heme oxygenase is believed to be the rate-limiting enzyme in heme degradation, the regulation of its activity and consequences of changes in its activity have received a great deal of attention

(reviewed in Ref. 21); the present findings with biliverdin reductase, however, indicate that this enzyme is not a passive component of the heme degradation pathway and that its activity potentially could become limiting in the elimination of heme degradation products.

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References

1. Singleton, J. W., and L. Laster. Biliverdin reductase of guinea pig liver. *J. Biol. Chem.* **240**:4780–4789 (1965).
2. Noguchi, M., T. Yoshida, and G. Kikuchi. Purification and properties of biliverdin reductase from pig spleen and rat liver. *J. Biochem. (Tokyo)* **86**:833–848 (1977).
3. Kutty, R. K., and M. D. Maines. Purification and characterization of biliverdin reductase from the rat liver. *J. Biol. Chem.* **256**:3956–3962 (1981).
4. Bell, J. E., and M. D. Maines. Kinetic properties and regulation of biliverdin reductase. *Arch. Biochem. Biophys.* **263**:1–9 (1988).
5. Huang, T. J., G. M. Trakshel, and M. D. Maines. Detection of ten variants of biliverdin reductase in rat liver by two-dimensional gel electrophoresis. *J. Biol. Chem.* **264**:7844–7849 (1989).
6. Huang, T. J., G. M. Trakshel, and M. D. Maines. Multiple forms of biliverdin reductase: modification of the pattern of expression in rat liver by bromobenzene. *Arch. Biochem. Biophys.* **270**:513–520 (1989).
7. Huang, T. J., G. M. Trakshel, and M. D. Maines. Microheterogeneity of biliverdin reductase in rat liver and spleen: selective suppression of enzyme variants in liver by bromobenzene. *Arch. Biochem. Biophys.* **274**:617–625 (1989).
8. Huang, T. J., and M. D. Maines. Bromobenzene-mediated alteration in activity and electrophoretic pattern of biliverdin reductase variants in rat kidney. *Mol. Pharmacol.* **37**:25–29 (1990).
9. Maines, M. D., G. M. Trakshel, and R. K. Kutty. Characterization of two constitutive forms of rat liver microsomal heme oxygenase: only one molecular species of the enzyme is inducible. *J. Biol. Chem.* **261**:411–419 (1986).
10. Cruse, I., and M. D. Maines. Evidence suggesting that the two forms of heme oxygenase are products of different genes. *J. Biol. Chem.* **263**:3348–3353 (1988).
11. Rotenberg, M. O., and M. D. Maines. Isolation, characterization and expression in *E. coli* of a cDNA encoding rat heme oxygenase-2. *J. Biol. Chem.* **265**:7501–7506 (1990).
12. Sun, Y., M. O. Rotenberg, and M. D. Maines. Developmental expression of heme oxygenase isozymes in rat brain: two HO-2 mRNAs are detected and HO-1 mRNA is inducible. *J. Biol. Chem.* **265**:8212–8217 (1990).
13. Thaler, M. M., D. L. Gemes, and A. F. Bakken. Enzymatic conversion of heme to bilirubin in normal and starved fetuses and newborn rats. *Pediatr. Res.* **6**:197–201 (1972).
14. Maines, M. D., and A. Kappas. Study of the developmental pattern of heme catabolism in liver and the effects of cobalt on cytochrome P-450 and the rate of heme oxidation during the neonatal period. *J. Exp. Med.* **141**:1400–1410 (1975).
15. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
16. Righetti, P. G., and J. W. Drysdale. Isoelectric focusing in gels. *J. Chromatogr.* **98**:271–321 (1974).
17. O'Farrell, P. H. High resolution two dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007–4021 (1975).
18. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203 (1981).
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
20. Trakshel, G. M., and M. D. Maines. Multiplicity of heme oxygenase isozymes: HO-1 and HO-2 are different molecular species in rat and rabbit. *J. Biol. Chem.* **264**:1323–1328 (1989).
21. Maines, M. D. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.* **2**:2557–2568 (1988).

Send reprint requests to: Mahin D. Maines, University of Rochester School of Medicine, Department of Biophysics, 601 Elmwood Ave., Rochester, NY 14642.