Reduced Nicotinamide–Adenine Dinucleotide Phosphate Dependent Biliverdin Reductase: Partial Purification and Characterization*

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ABSTRACT: An enzyme capable of converting biliverdin into bilirubin was identified in the soluble fraction of various rat and guinea pig tissues. The enzyme was purified 52-fold by successive ammonium sulfate fractionation, calcium phosphate gel adsorption, Sephadex G-200 filtration, and chromatography on DEAE-cellulose. The enzyme has an absolute requirement for reduced pyridine nucleotide; its activity with reduced nicotinamide–adenine dinucleotide phosphate is over 50 times greater than with reduced nicotinamide–adenine dinucleotide.

Enzyme activity is increased by low concentrations of human albumin; albumin concentrations in excess of 2 mg/ml of incubation mixture are inhibitory. Trypsin, *p*-hydroxymercuribenzoate, and copper ions, and to a lesser degree lipase, iodoacetate, bilirubin, and ferrous, and ferric ions, inhibit the enzyme. Reduced nicotinamide–adenine dinucleotide phosphate dependent biliverdin reductase, coupled with microsomal heme oxygenase, converts hemin into bilirubin; in this system, biliverdin reductase is not rate limiting. The kinetics and tissue distribution of the heme oxygenase–biliverdin reductase system suggest that it plays a major role in the physiologic degradation of hemoglobin to bile pigment.

In the intact organism, administered hemoglobin or hematin is converted almost quantitatively into bilirubin and carbon monoxide (Ostrow *et al.*, 1962; Coburn *et al.*, 1963). We recently described an enzyme system present in the spleen, liver, and other tissues that converts heme into bilirubin (Tenhunen *et al.*, 1968); its activity *in vitro* agrees well with the kinetic requirements for the physiologic turnover of hemoglobin in the intact organism. The system consists of a microsomal mixed-function oxygenase that cleaves the ferriprotoporphyrin ring of methemoglobin or of isolated α or β chains of hemoglobin to form biliverdin (Tenhunen *et al.*, 1969b). The present studies demonstrate that the biliverdin so produced is then reduced to bilirubin by a soluble, heat-labile, nondialyzable factor that has the properties of an NADPH-dependent reductase (Tenhunen *et al.*, 1968).

It has long been assumed that biliverdin is an intermediate in the physiologic conversion of heme into bilirubin (Lemberg, 1956). *In vivo*, isotopically labeled biliverdin is rapidly reduced to bilirubin (Goldstein and Lester, 1964). *In vitro*, coupled oxidation of certain heme-containing compounds with ascorbic acid and molecular oxygen leads to formation of several poorly defined derivatives from which small amounts of biliverdin may be obtained upon treatment with acetic acid (Lemberg, 1956). However, this nonenzymatic model system probably does not represent the physiologic mechanism for bile pigment formation (Petryka *et al.*, 1962; Nichol and Morell, 1969).

In 1936, Lemberg and Wyndham reported the qualitative conversion of biliverdin into bilirubin by minced guinea pig liver. This observation, which was consistent with an enzymatic reduction, was ascribed to the presence of several nonspecific dehydrogenases. In 1965, Singleton and Laster described a soluble enzyme present in guinea pig liver and in several other tissues that catalyzed the reduction of biliverdin. The enzyme was purified 15-fold and had an absolute requirement for reduced pyridine nucleotide; NADH was much more active than NADPH. By contrast, the biliverdin reductase detected in association with the recently characterized heme oxygenase (Tenhunen *et al.*, 1968) has an absolute and stoichiometric requirement for NADPH. The present report describes the partial purification and some of the functional properties of NADPH-dependent biliverdin reductase.

Experimental Section

Materials. NADPH, NADH, and lipase (type II) were obtained from Sigma Chemical Co., sterile trypsin (3 × crystallized) from Worthington Biochemical Corp., Sephadex G-200 from Pharmacia Fine Chemicals, calcium phosphate gel from Bio-Rad Laboratories, Whatman DEAE-cellulose (DE 52) from H. Reeve Angel and Co., Aquacide 1 (mol wt 70,000) from Calbiochem, and human serum albumin (crystalline) from Pentex, Incorporated. Biliverdin was prepared by oxidizing crystalline bilirubin (Pfanstiehl Chemical Co.) with $FeCl_3$ in hydrochloric acid (Gray *et al.*, 1961). In methanolic solution this material displayed absorption maxima at 375 and 656 mμ with a ratio between the two bands of 3.10–3.13 (Gray *et al.*, 1961; Goldstein and Lester, 1964). Biliverdin was dissolved in methanol and 10–20 μl of this solution equivalent to approximately 0.06 μmole of the pigment was used for the incubations.

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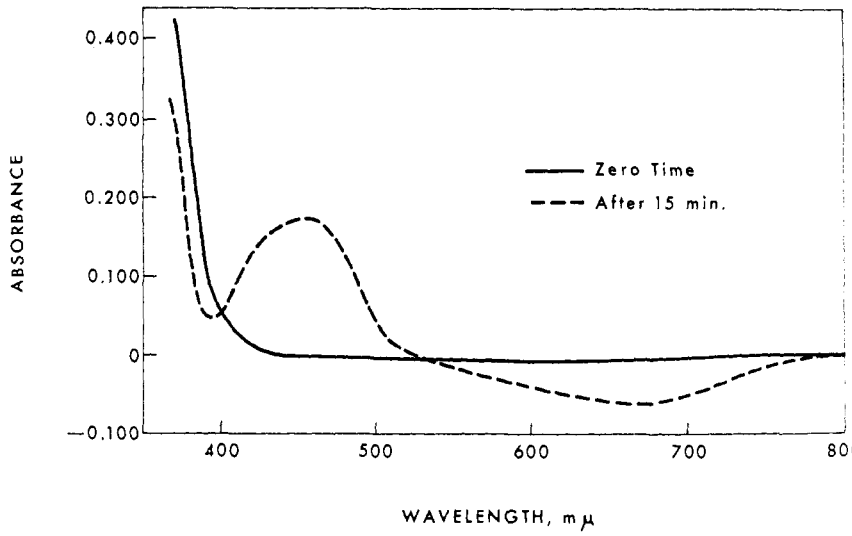

FIGURE 1: Conversion of biliverdin into bilirubin by biliverdin reductase. The difference spectra demonstrate bilirubin formation and biliverdin disappearance in the incubation mixture containing step 3 enzyme after 15 min at 37°. The sample cuvet contained the incubation mixture (3.0 ml) of 0.2 mg of enzyme protein, 20 μM biliverdin, 90 μM NADPH, and 95 mM potassium phosphate buffer (pH 7.4). In the reference cuvet, NADPH was replaced by 0.1 M potassium phosphate buffer (pH 7.4).

Methods. For the standard enzyme assay procedure the following conditions were chosen. The final assay mixture contained 95 mM potassium phosphate buffer (pH 7.4), 90 μM NADPH, 20 μM biliverdin, and 0.035% human serum albumin in a total volume of 3.0 ml. In the control cuvet NADPH was replaced by 0.1 M potassium phosphate buffer (pH 7.4). The complete reaction mixtures minus the NADPH or the 0.1 M potassium phosphate buffer were preincubated for 5 min at 37°. After addition of the NADPH or the phosphate buffer, the reaction rate was monitored for 15–20 min in a Gilford Model 2000 spectrophotometer equipped with a constant-temperature cuvet chamber set at 37°. Formation of bilirubin was calculated from the increase in optical density at 460 mμ, at which wavelength the pigment absorbed maximally in the incubation mixture used. A unit of enzyme activity was defined as that amount of enzyme which resulted in the formation of 1 mμmole of bilirubin/min.

The conversion of biliverdin into bilirubin was demonstrated by use of a Shimadzu MPS-50L split-beam recording spectrophotometer (Figure 1). The difference in spectrum between the sample cuvet containing enzyme protein, biliverdin, buffer, and NADPH was read against a reference cuvet from which only NADPH was omitted. Validation of this assay is described in the section on Results. The extinction coefficients of bilirubin and biliverdin were determined as discussed previously (Tenhunen *et al.*, 1968). For bilirubin the millimolar extinction coefficient, at 460 mμ, ranged from 27.5 to 32.7 and for biliverdin, at 650–660 mμ, from 7.3 to 8.0. Bilirubin formed in the incubation mixture was identified by its spectral properties, by spectrophotometric determination of the pigment after chloroform extraction, and by thin-layer chromatography on silica gel, using a solvent system consisting of methyl ethyl ketone–propionic acid–water (20:5:5) (Schmid, 1957; Tenhunen, 1965). NADPH consumption was estimated by the decrease in optical density at 340 mμ. The $\Delta\epsilon_{mM}$ of this reaction was 19.2 ($\Delta\epsilon_{mM\ NADPH-NADP} + \Delta\epsilon_{mM\ biliverdin-bilirubin}$). Enzyme activities were calculated from maximum reaction rates. Chloride determinations were performed by the method of Schales and Schales (1941). Protein was quantitated by the method of Lowry *et al.* (1951). Additional details of experimental procedures are given in the legends to the tables and figures.

In all instances, nonfasted male Sprague–Dawley rats weighing 300–400 g were used.

Results

Enzyme Purification. Immediately after decapitation of the rats the liver was perfused through the portal vein with cold isotonic saline, and the liver and kidneys were homogenized in 2–3 volumes (w/v) of 0.25 M cold sucrose containing 0.05 M Tris-HCl buffer (pH 7.5). The homogenates were fractionated by standard procedures (Schneider, 1948) or by modifications specifically noted in the text. All of the subsequent preparative procedures were carried out at 0 to 4°. Although the specific activity of biliverdin reductase was highest in the 105,000*g* supernatant fraction, this did not differ significantly from that of the supernatant after centrifugation at 30,000*g* for 30 min. For practical reasons the purification procedure described below was performed on the 30,000*g* supernatant of the kidneys of 18 rats (step 1) (Table I).

Solid ammonium sulfate was added slowly to the kidney supernatant to make it a 40% saturated solution. After 30 min the preciptate was removed by centrifugation at 10,000*g* for 10 min and the supernatant was brought to 60% saturation with additional ammonium sulfate (step 2). The precipitate was collected by centrifugation, taken up in a small volume of 0.01 M potassium phosphate buffer (pH 7.4), and dialyzed against distilled water for 24 hr in Visking tubing prewashed once with 1 mM EDTA and twice with distilled water. During dialysis a protein precipitate appeared. It was devoid of enzymatic activity and was removed by centrifugation at 10,000*g* for 10 min (step 3).

The clear supernatant was diluted with 0.01 M potassium phosphate buffer (pH 7.0), to a protein concentration of 10 mg/ml. Calcium phosphate gel (80 mg/ml), equivalent to 12 mg/mg of protein, was added to the solution and stirred occasionally for 30 min, and the mixture was centrifuged at 10,000*g* for 10 min. The supernatant was discarded. The gel was washed once with 1 volume of distilled water and the enzyme then was eluted with one volume of 0.03 M potassium phosphate buffer (pH 7.4) (step 4). The clear supernatant obtained by centrifugation (10,000*g*, 10 min) was dehydrated in dry Aquacide 1 to a small volume.

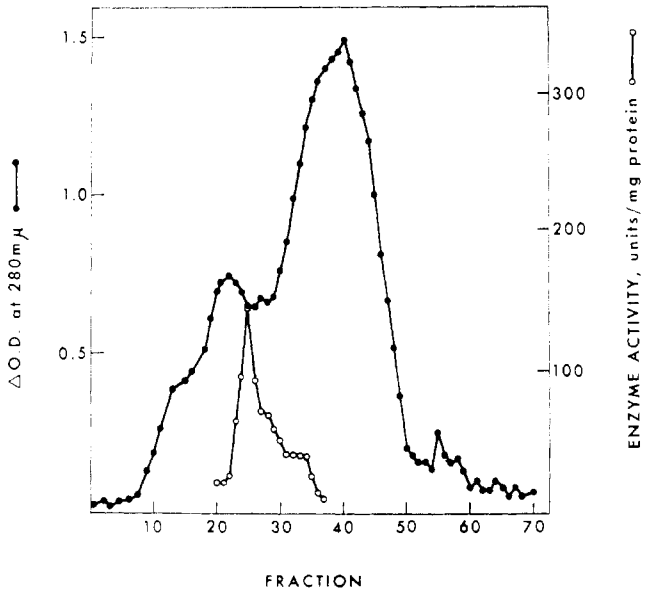

FIGURE 2: Elution pattern of biliverdin reductase from Sephadex G-200. Concentrated protein (125 mg; specific activity 27.0) from step 4 was applied to a column of Sephadex G-200 and eluted in Tris-HCl buffer, 0.05 M (pH 7.0), in 6.0-ml fractions. The absorbance at 280 mμ and enzyme activity are shown.

The concentrated clear solution was applied to a Sephadex G-200 column (35 × 3 cm), previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.0). The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min and appeared immediately behind the first protein peak (step 5) (Figure 2). The fractions containing the highest activity were pooled, dehydrated in dry Aquacide to a small volume, and dialyzed for 4–6 hr against an aqueous solution of 10 mM Tris-HCl (pH 7.0) and 10 mM EDTA.

The concentrated enzyme preparation was subsequently applied to a DEAE-cellulose column (25 × 2 cm), which was prepared in 10 mM Tris-HCl (pH 7.0) and 10 mM EDTA and first washed with 150 ml of the same buffer. The column was developed with a 400-ml gradient from 10 mM Tris-HCl (pH 7.0) and 10 mM EDTA to 0.25 M KCl and 10 mM EDTA (step 6) (Figure 3).

Similar degrees of purification were obtained with kidney and liver homogenates and both enzyme preparations were comparable in stability and kinetic properties. Further purification of the enzyme preparations was attempted by adsorption on hydroxylapatite, additional ammonium sulfate precipitation and Sephadex G-200 filtration. However, no additional purification was achieved or enzyme activity was entirely lost.

Stability of the Enzyme. During 8 weeks of preservation, enzyme preparations after purification steps 1, 2, 3, and 5, containing 1 to 10 mg of protein per ml, lost 5–25% of activity at −20°, 15–45% at 0°, and 90–100% at 4°. Rapid freezing and thawing did not significantly alter the activity. After calcium

TABLE I: Specific Activity of Biliverdin Reductase of Rat Kidney at Various Stages of Purification.

Steps of Purification	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Purification Factor
(1) 30,000*g* supernatant	9302	2448	3.8	1
(2) 40–60% ammonium sulfate precipitation	6953	818	8.5	2
(3) Dialysis	6219	527	11.8	3
(4) Calcium phosphate gel adsorption	3375	125	27.0	7
(5) Sephadex G-200 filtration	1734	21.2	81.8	22
(6) DEAE-cellulose chromatography	1107	5.6	197.7	52

TABLE II: Relationship between Biliverdin Disappearance, Bilirubin Formation, and NADPH Consumption.[a]

Expt	mμmoles/min per mg of Protein		
	Biliverdin Disappearance	Bilirubin Formation	NADPH Consumption
1	126	123	92
2	156	135	112
3	75	78	52
4	81	69	50

[a] Biliverdin disappearance, bilirubin formation, and NADPH consumption in the standard incubation mixture were measured as described in the text employing step 5 preparations as the enzyme source. Values of four individual determinations are given.

TABLE III: Biliverdin Reductase Activity of Different Rat Tissues.[a]

Tissue	Specific Activity (units/mg)	
	NADPH	NADH
Liver	1.50	0.005
Kidney	3.12	0.001
Spleen	3.65	0.011
Brain	1.06	Not measurable
Muscle (abdominal wall)	0.12	0.001

[a] The tissues were homogenized in 2–3 volumes of 0.25 M sucrose (w/v), and centrifuged 10 min at 10,000*g*, 10 min at 20,000*g*, and 60 min at 40,000*g*. The supernatant of the last centrifugation was employed for the enzyme assay which was performed as described in the text. The concentration of NADPH or NADH was 90 μM. Specific activity units are mμmoles of bilirubin formed per min.

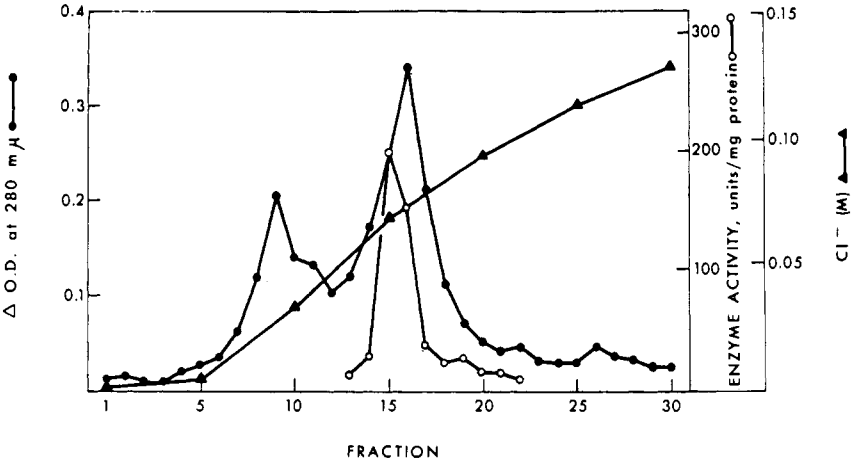

FIGURE 3: Elution pattern of biliverdin reductase from DEAE-cellulose. Dialyzed protein (21.2 mg; specific activity 81.8) from step 5 was applied to a column of DEAE-cellulose and eluted in a gradient of 10 mM Tris-HCl (pH 7.0)–10 mM EDTA to 0.25 M KCl–10 mM EDTA, in 6.0-ml fractions, as described in the text. The absorbance at 280 mμ, enzyme activity and the Cl^- gradient are shown.

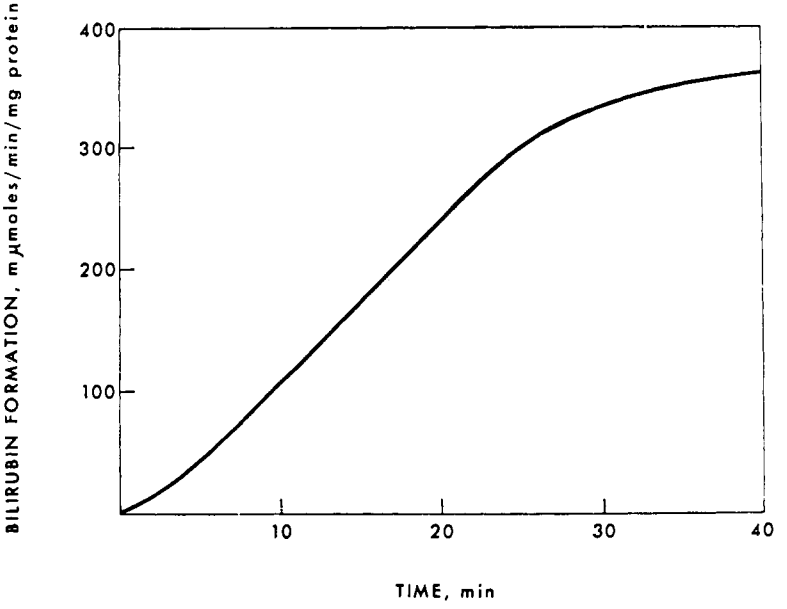

FIGURE 4: Continuous recording of the conversion of biliverdin into bilirubin by biliverdin reductase as a function of time. Reaction mixture (3.0 ml) consisted of step 3 enzyme (0.5 mg of protein), 20 μM biliverdin, 90 μM NADPH, and 95 mM potassium phosphate buffer (pH 7.4). In the control cuvet, NADPH was replaced by 0.1 M potassium phosphate buffer (pH 7.4).

phosphate gel treatment (step 4) the enzyme preparations were less stable, particularly at 4°, and hence were processed rapidly. On freezing overnight, loss of activity did not exceed 10%. After purification step 5, enzyme activity was completely lost at 56° within 5 min.

Identification of Reaction Product. The following evidence indicated that the reaction product exhibiting an absorption maximum at 460–470 mμ (Figure 1) was bilirubin formed from the added substrate, biliverdin: (a) When crystalline bilirubin was added to the incubation mixture used, a similar absorption band at 460–470 mμ was observed. (b) When a methanolic solution of diazotized sulfanilic acid was added directly to the incubation mixture, a red azoderivative characteristic of bilirubin (Malloy and Evelyn, 1937; Lemberg and Legge, 1949) was formed only when the enzyme had been incubated with biliverdin and NADPH. (c) After incubation with biliverdin, a yellow pigment was extractable that had the solubility and spectrophotometric properties of bilirubin and gave a positive diazo reaction (Malloy and Evelyn, 1937; Lemberg and Legge, 1949). (d) On chromatography on silica gel, this yellow chloroform-soluble pigment had an R_F of 0.70 which was identical with that of authentic bilirubin. (e) Almost all of the biliverdin that disappeared on incubation was accounted for by the bilirubin formed (Table II).

Characteristics of the Enzyme Preparation. The enzymatic conversion of biliverdin into bilirubin required NADPH, which on an equimolar basis could be replaced only to a very small extent by NADH (Table III). Even a 50-fold increase in the NADH concentration resulted in only half the maximal NADPH activity. The rate of bilirubin formation from biliverdin was maximal and constant for about 15 min with the enzyme concentrations used (Figure 4). As shown in Figure 5, bilirubin formation was directly proportional to the concentration of enzyme, from 0.02 to 0.25 mg of protein per ml of the incubation mixture.

The reaction rate was enhanced by addition of small amounts of human serum albumin to the incubation mixture (Figure 6). An increase of up to 40% was observed in the presence of 0.3–1.0 mg of albumin/ml, but albumin concentrations in excess of 2 mg/ml were inhibitory (Figure 6). In the standard enzyme assay, 0.3 mg of albumin/ml was used.

The effect of pH on biliverdin reductase activity was ex-

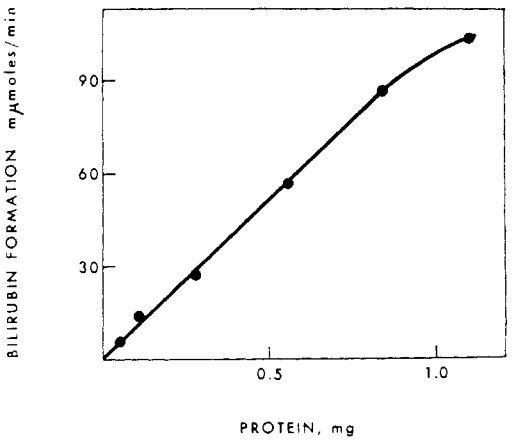

FIGURE 5: Bilirubin formation as a function of enzyme concentration. Step 5 enzyme was assayed for biliverdin reductase activity under standard conditions.

amined with an enzyme preparation obtained by step 3 purification. In 0.1 M potassium phosphate buffer the reaction velocity was optimal between pH 7.0 and 7.4. The apparent K_m for biliverdin reductase, using a step 5 purified preparation, was calculated from a Lineweaver–Burk plot (Lineweaver and Burk, 1934) (Figure 7); the value obtained was 3.7 μM.

Stoichiometry. Stoichiometric measurements were made in the standard assay procedure with a step 5 purified preparation. In four individual assays, for each mole of biliverdin utilized, 1 mole of bilirubin was formed and 0.7–0.8 mole of NADPH was consumed (Table II). This closely approximates the value of 1 characteristic of reactions catalyzed by NADPH-dependent reductases. The principal source of error in this determination is the large contribution of the $\Delta\epsilon_{mM\ biliverdin-bilirubin}$ (12.9). This change in absorbancy at 340 mμ is even greater for the overall conversion of heme into bilirubin and has thereby complicated assay of NADPH consumption during microsomal heme oxygenation (Tenhunen *et al.*, 1969a–c).

Inhibition. In the standard assay procedure, minimal or no inhibitory effect was observed by addition of the following substances to the incubation mixture: EDTA (1×10^{-3} M), NaN_3 (1×10^{-3} M), KCN (1×10^{-3} M), iodoacetate (1×10^{-3} M), *o*-phenanthroline (1×10^{-3} M), Fe^{2+} ($FeSO_4$) or Fe^{3+} ($FeCl_3$) (1×10^{-4} M), and Mg^{2+} (1×10^{-3} M). Fe^{2+} (1×10^{-3} M) inhibited 32% and Fe^{3+} (1×10^{-3} M) 19%. *p*-Hydroxymercuribenzoate inhibited 14% at 1×10^{-6} M, 88% at 1×10^{-5} M, and completely at 1×10^{-4} M. Copper ($CuSO_4$) inhibited 91% at a concentration of 1×10^{-4} M and completely at 1×10^{-3} M. The inhibitory effect of trypsin in a concentration of 0.1 mg/ml was 22%, with 0.2 mg/ml 59% and with 0.4 mg/ml 85%. Lipase inhibited as follows: in a concentration of 0.1 mg/ml, 19%; 0.2 mg/ml, 27%; and 0.4 mg/ml, 36%. At least in part, lipase inhibition appeared to be accounted for by the small trypsin impurities present in the lipase preparation used. These inhibitory effects of trypsin and lipase were obtained by the addition of the enzymes at the beginning of the routine biliverdin reductase assay. No attempts were made to determine the effects of preincubation of biliverdin reductase with proteolytic or lipolytic enzymes. Bilirubin was slightly inhibitory for biliverdin reductase; in a concentration of 17 μM the reaction rate was reduced 20% and with 86 μM, approximately 50%. In the standard incubation procedure product inhibition did not appear to be of significance, because the final bilirubin concentration seldom exceeded 4 μM.

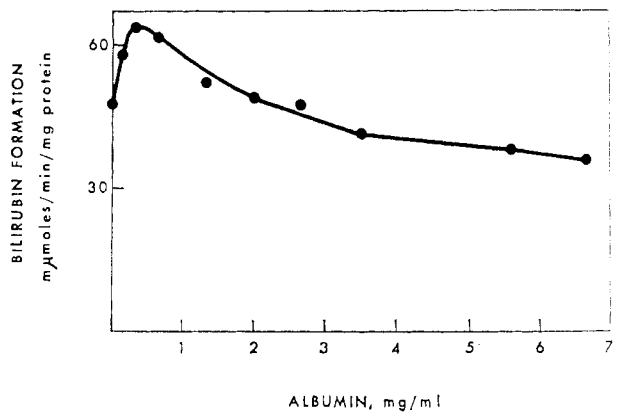

FIGURE 6: Effect of albumin on the reaction rate. Step 5 enzyme (22 μg of protein) was used under standard conditions of enzyme assay with different amounts of crystalline human serum albumin.

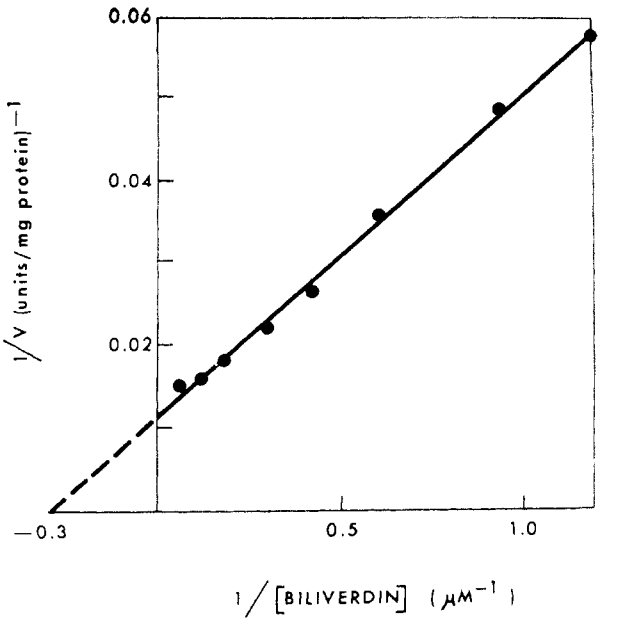

FIGURE 7: Reaction rate as a function of substrate concentration Step 5 enzyme (6.5 μg of protein) was assayed for biliverdin reductase activity under standard conditions.

Discussion

The present study demonstrates the existence of a factor in the soluble portion of various tissues that is capable of converting biliverdin into bilirubin. That this factor is an enzyme is suggested by its catalytic properties, its absolute requirement for NADPH, its irreversible inhibition by trypsin, its heat lability, and its macromolecular nature as evidenced by its behavior during the various purification procedures.

These findings confirm that the enzymatic conversion of heme into bilirubin is a two-step process. In the first step heme is

transformed to biliverdin by microsomal heme oxygenase which appears to be a mixed-function oxidation requiring NADPH and molecular oxygen (Tenhunen *et al.*, 1968). This reaction is coupled with a soluble, NADPH-dependent biliverdin reductase which forms bilirubin. While heme oxygenase may be present in high concentrations only in the spleen and liver (Tenhunen *et al.*, 1969a–c), NADPH-dependent biliverdin reductase is more widely distributed (Table III). It can be calculated from the measured activities of the enzyme in different tissues (Table III) that it is present in great excess in relation to microsomal heme oxygenase. Both enzymes have a similar apparent K_m; this is 5.0 μM for microsomal heme oxygenase in a crude microsomal preparation and 3.7 μM for biliverdin reductase in a partially purified system.

Recent studies (R. Tenhunen, H. S. Marver, and R. Schmid, 1969, unpublished data) showed that the biliverdin produced by heme oxygenase of spleen homogenate consists largely of the α isomer, suggesting the enzyme's physiologic role in heme catabolism. The isomer specificity of NADPH-dependent biliverdin reductase cannot be assessed because the only biliverdin isomer available for testing is of the α configuration.

The present enzyme preparation obtained from rat liver and kidney resembles in overall activity and in tissue distribution that reported by Singleton and Laster (1965), derived from guinea pig liver. Important differences were noted, however, between the two preparations. At all stages of purification, enzyme activity was dependent on the presence of NADPH, whereas with the preparation of Singleton and Laster, NADH was significantly more effective. Moreover, while with the enzyme of Singleton and Laster, concentrations of albumin up to 40 mg/ml in the incubation mixture increased activity fourfold, in the present study, albumin in excess of 2 mg/ml was inhibitory. These discrepancies cannot be explained by species differences because with guinea pig tissues, we obtained results similar to those in rat.

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References

Coburn, R. F., Blakemore, W. S., and Foster, R. E. (1963), *J. Clin. Invest. 42*, 1172.

Goldstein, G. W., and Lester, R. (1964), *Proc. Soc. Exptl. Biol. Med. 117*, 681.

Gray, C. H., Lichtarowicz-Kulczycka, A., Nicholson, D. C., and Petryka, Z. (1961), *J. Chem. Soc.*, 2264.

Lemberg, R. (1956), *Rev. Pure Appl. Chem. 6*, 1.

Lemberg, R., and Legge, J. W. (1949), *in* Hematin Compounds and Bile Pigments, New York, N. Y., Interscience, pp 114–123.

Lemberg, R., and Wyndham, R. A. (1936), *Biochem. J. 30*, 1147.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Malloy, H. T., and Evelyn, K. A. (1937), *J. Biol. Chem. 119*, 481.

Nichol, A. W., and Morell, D. B. (1969), *Biochim. Biophys. Acta 184*, 173.

Ostrow, J. D., Jandl, J. H., and Schmid, R. (1962), *J. Clin. Invest. 41*, 1628.

Petryka, Z., Nicholson, D. C., and Gray, C. H. (1962), *Nature 194*, 1047.

Schales, O., and Schales, S. S. (1941), *J. Biol. Chem. 140*, 879.

Schmid, R. (1957), *J. Biol. Chem. 229*, 881.

Schneider, W. C. (1948), *J. Biol. Chem. 176*, 259.

Singleton, J. W., and Laster, L. (1965), *J. Biol. Chem. 240*, 4780.

Tenhunen, R. (1965), *Ann. Med. Exptl. Fenniae (Helsinki) 43, Suppl. 6.*

Tenhunen, R., Marver, H. S., and Schmid, R. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 748.

Tenhunen, R., Marver, H. S., and Schmid, R. (1969a), *Clin. Res. 17*, 467.

Tenhunen, R., Marver, H. S., and Schmid, R. (1969b), *Trans. Assoc. Am. Physicians* (in press).

Tenhunen, R., Marver, H. S., and Schmid, R. (1969c), *J. Biol. Chem.* (in press).