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Bilirubin inhibition of enzymes involved in the mitochondrial malate-aspartate shuttle

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The effect of increasing bilirubin concentrations upon the catalytic activity of a series of dehydrogenases and aminotransferases was examined. The particular enzymes were chosen to examine the effect of bilirubin upon the activity of enzymes responsible for the indirect transfer of reducing equivalents across the inner mitochondrial membrane. Malate dehydrogenase was inhibited at very low concentrations of bilirubin and showed competitive inhibition with respect to coenzyme of 2 μM, while the cytosolic form of this enzyme exhibited a 15 μM inhibition constant. Cytosolic glycerol-3-phosphate dehydrogenase was not appreciably inhibited by bilirubin. Both the mitochondrial and cytosolic forms of aspartate aminotransferase showed moderate competitive bilirubin inhibition with respect to substrates with a K_i of 30 μM with respect to 2-oxoglutarate and a K_i of 80 μM with respect to aspartate. Preincubation studies indicated that inhibition was reversible for all enzymes examined. These results are interpreted in terms of the inhibition of the malate-aspartate shuttle by relatively low concentrations of bilirubin.

Introduction

Bilirubin, a tetrapyrrole-based bile pigment, is a catabolic product of hemoproteins and is extremely toxic to cells [1,2]. A number of excellent reviews concerning bilirubin structure, metabolism and toxicity have been published [3,4]. High serum concentrations of bilirubin are responsible for the pathogenesis of jaundice or icterus. Bilirubin is extremely toxic to newborn infants and can induce the clinical condition known as kernicterus [2,5]. Bilirubin has been shown to cause an inhibition of respiration in rat brain homogenates [6] and appears to have an extremely detrimental effect on membrane-requiring mitochondrial reactions. At concentrations of approx. 3 μM, bilirubin has been shown to induce an energy-dependent irreversible swelling of the mitochondria, abolition of respiratory control and a large decrease in the phosphorylation/oxygen ratio [7,8]. Bilirubin uncouples oxidative phosphorylation in mitochondria [9–11] and has also been shown to interact with mitochondrial lipids [12]. Protein phosphorylation in cerebral cell-free preparations has also been shown to be inhibited by bilirubin [13]. Although much of this toxic response appears to be due to non-specific disruption of membrane structure, the mechanism of bilirubin toxicity has not been totally explained on a molecular basis. It appears probable that at least part of the bilirubin toxicity may be due to direct interactions with specific proteins or with specific components of

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the mitochondrial membrane.

Bilirubin has been shown to inhibit several individual enzymes. Relatively low concentrations of bilirubin have been shown to inhibit glutathione S-transferase [14], protein kinase [13,15], alcohol dehydrogenase [16], NAD-linked isocitrate dehydrogenase [17], cholesterol esterase [18], trypsin and chymotrypsin [19] as well as bind to α-foetoprotein [20]. Initial studies on the mitochondrial enzymes glutamate dehydrogenase, malate dehydrogenase and NADH-dehydrogenase have shown inhibition by bilirubin of all of their activities [21–23]. In spite of all of these studies the mechanism of toxicity by bilirubin is poorly understood and no evidence of inhibition of a distinct metabolic pathway by the interaction of bilirubin with specific enzymes has been presented to date.

Intact mammalian mitochondria are unable to oxidize cytosolic NADH. In mammals the reducing equivalents enter the mitochondria indirectly by means of coordinated reactions catalyzed by cytosolic and mitochondrial isozymes. The most prevalent of these mechanisms appears to be the malate-aspartate shuttle although in some tissues the glycerol phosphate shuttle may be significant [24]. We wish to report here a comparative study of the bilirubin inhibition of several cytosolic and mitochondrial enzymes and isoenzymes. In particular we wish to report the relative inhibition by bilirubin of enzymes involved in the metabolic shuttle pathway responsible for the transport of reducing equivalents across the inner mitochondrial membrane.

Materials and Methods

Microcrystalline L-3-hydroxyacyl coenzyme A dehydrogenase [EC 1.1.1.35] prepared from porcine heart [25] was generously provided by the laboratory of L.J. Banaszak, Washington University and was separated from the polyethylene glycol crystallization media by passage through a 0.7 × 20 cm Bio-Gel P-30 column before use. This enzyme was assayed under conditions described by Noyes and Bradshaw [26]. Purified porcine heart mitochondrial aspartate aminotransferase [EC 2.6.1.2] was a generous gift from the laboratory of M. Martínez-Carrion, Virginia Commonwealth University, and was assayed by a coupled enzyme system using excess cytosolic malate dehydrogenase as the auxiliary enzyme as described by Okamoto and Murino [27]. Porcine heart mitochondrial malate dehydrogenase [EC 1.1.1.37] was purified by the method of Eberhardt and Wolfe [28] and porcine heart cytosolic malate dehydrogenase was purified by a modification of the method of Gerding and Wolfe [29]. This modification involved the use of Affi-Gel Blue (Bio-Rad Laboratories) affinity chromatography in place of the hydroxyapatite chromatography step [30]. Bovine liver glutamate dehydrogenase, porcine heart cytosolic aspartate aminotransferase, porcine heart alanine aminotransferase, and rabbit muscle glycerol phosphate dehydrogenase were obtained in purified form from Sigma Chemical Company and were used without further treatment or purification.

The substrate S-acetoacetyl panthethine was synthesized by the method of Noyes and Bradshaw [26]. All other enzyme substrates and coenzymes were obtained from Sigma Chemical Company. Bilirubin stock solutions were prepared by dissolving a weighed amount of the pure solid (Sigma) in 0.25 ml of 0.1 M NaOH and diluting to the appropriate volume with buffer. These solutions were stored in the dark and were used within 6 h of their preparation. All solutions were prepared from reagent grade chemicals in deionized water. All buffers contained 1.0 mM EDTA and 1.0 mM 2-mercaptoethanol. Assay conditions for each individual enzyme were performed in accordance with established methods and are defined in the figure legends. Only small quantities of the bilirubin stock solutions were added to the buffered assays so that the pH of the assay solutions was not appreciably affected. Rate measurements were carried out spectrophotometrically by observing the rate of oxidation of NADH at 340 nm. All experiments were performed in polystyrene cuvettes in order to eliminate experimental difficulties due to quartz and glass surface absorption of bilirubin [31].

Results

The effect of increasing concentration of bilirubin on the catalytic activity of selected

mitochondrial and cytosolic (supernatant) dehydrogenases is shown in Fig. 1. The extent of inhibition varied with each dehydrogenase studied. The malate dehydrogenase isozymes were strongly inhibited with the mitochondrial form of malate dehydrogenase showing inhibition at extremely low levels of bilirubin and exhibiting an I_{50} value of 3 μM under the assay conditions chosen for study. It is also notable that glycerol-3-phosphate dehydrogenase, an enzyme involved in a separate and distinct metabolic pathway for the shuttle of reducing equivalents across the mitochondrial barrier, was not appreciably inhibited at even the highest attainable concentrations of bilirubin studied. The enzyme 3-hydroxyacyl coenzyme A dehydrogenase is only slightly inhibited at high concentrations of bilirubin. This enzyme has been shown to be very similar to mitochondrial malate dehydrogenase with respect to its cellular location, type of reaction catalyzed [32], mechanism of reaction [33], and secondary, tertiary and quaternary structure [34].

The effect of increasing concentration of bilirubin on the catalytic activity of the mitochondrial and cytosolic (supernatant) isozymes of aspartate aminotransferase and upon alanine aminotransferase is shown in Fig. 2 The extent of inhibition for each of these enzymes is approximately the same under the conditions chosen for study. The extent of inhibition is far less than that for the malate dehydrogenase isozymes and appears to be independent of whether the enzyme was isolated from a cytosolic or mitochondrial source. The aspartate aminotransferases are inhibited approximately the same as alanine aminotransferase, a cytosolic enzyme not directly involved in the shuttle of reducing equivalents into the mitochondria.

In order to determine the mode of, and the extent of inhibition by bilirubin, the inhibition kinetics of the mitochondrial and cytosolic isozymes of malate dehydrogenase and aspartate aminotransferase were further examined. Figs. 3 and 4 are classical Dixon plots [35] for the bilirubin inhibition with respect to reduced coenzyme

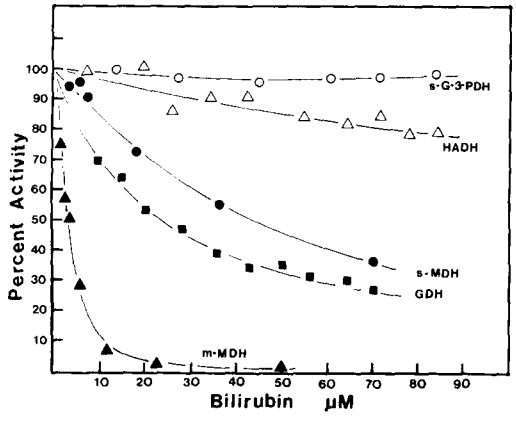

Fig. 1. Inhibition of selected dehydrogenases by bilirubin. Plot of percentage initial activity vs. micromolar concentration of bilirubin. Assay conditions are as follows: s-Glycerol-3-phosphate dehydrogenase (s-G-3-PDH) (○———○) 300 μM dihydroxyacetone phosphate, 100 μM NADH (pH 7.0), L-3-hydroxyacyl coenzyme A dehydrogenase (HADH) (△———△) 100 μM S-acetoacyl panthethine, 100 μM NADH (pH 7.0), s-malate dehydrogenase (s-MDH) (●———●) and m-malate dehydrogenase (m-MDH) (■———■) 100 μM oxaloacetate, 20 μM NADH (pH 8.0), glutamate dehydrogenase (GDH) (▲———▲) 0.5 mM 2-oxoglutarate, 20 mM NH_4Cl, 100 μM NADH (pH 7.5). All assays were performed in Tris buffer.

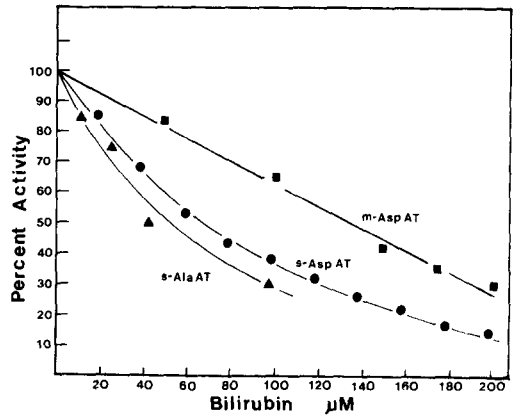

Fig. 2. Inhibition of selected aminotransfereses by bilirubin. Plot of percentage initial activity vs. micromolar concentration of bilirubin. Assay conditions are as follows: m-aspartate aminotransferase (m-AspAT) (■———■) 2.0 mM 2-oxoglutarate, 0.50 mM aspartate, excess s-malate dehydrogenase: s-aspartate aminotransferase (s-AspAT) (●———●) 3.0 mM 2-oxoglutarate, 10 mM aspartate: excess s-malate dehydrogenase: s-alanine aminotransferase (s-AlaAT) (▲———▲) 10 mM 2-oxoglutarate, 200 mM alanine, excess lactate dehydrogenase. All assays were performed in Tris buffer (pH 8.3) and contained 0.1 mM NADH.

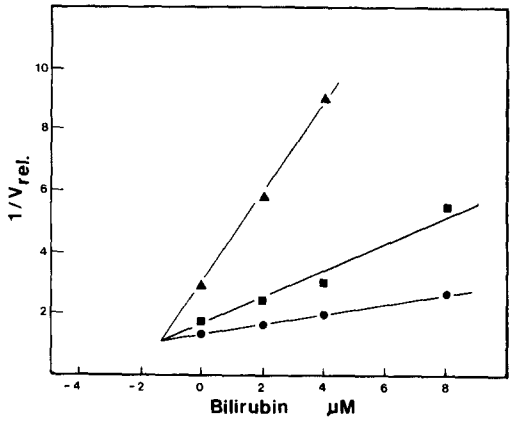

Fig. 3. Inhibition of mitochondrial malate dehydrogenase by bilirubin. Plot of inverse relative reaction velocity vs. micromolar concentration of bilirubin at various concentrations of coenzyme NADH. The oxaloacetate concentration was constant at 200 µM. The concentrations of NADH are as follows: 20 µM (▲———▲), 40 µM (■———■), 100 µM (●———●). Lines intersect at $-K_i$ of approx. 2 µM.

concentration for the mitochondrial and cytosolic forms of malate dehydrogenase. These plots demonstrate that inhibition is competitive with respect to coenzyme for both forms of this enzyme and that the inhibition constants are low: 2 and 15 µM

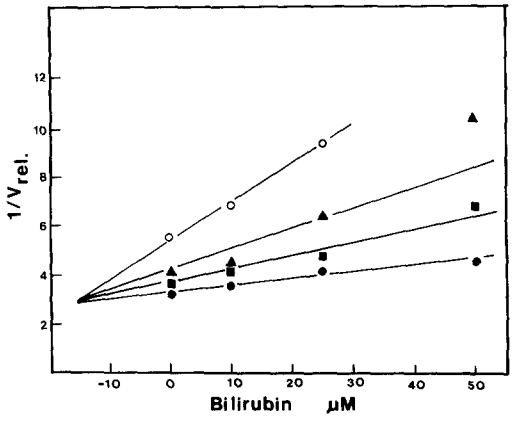

Fig. 4. Inhibition of cytosolic (supernatant) malate dehydrogenase by bilirubin. Plot of inverse relative reaction velocity vs. micromolar concentration of bilirubin at various concentrations of coenzyme NADH. The oxaloacetate concentration was constant at 200 µM. The concentrations of NADH are as follows: 12.5 µM (○———○), 17 µM (▲———▲), 25 µM (■———■), 50 µM (●———●). Lines intersect at $-K_i$ of approx. 15 µM.

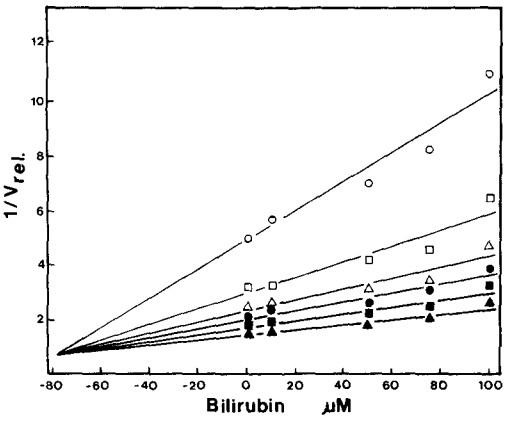

Fig. 5. Inhibition of cytosolic (supernatant) aspartate aminotransferase by bilirubin. Plot of inverse relative reaction velocity vs. micromolar concentration of bilirubin at various concentrations of the substrate aspartate. The concentration of 2-oxoglutarate was constant at 0.667 mM. The concentrations of aspartate are as follows: 0.833 mM (○———○), 1.67 mM (■———□), 2.50 mM (△———△), 3.33 mM (●———●), 5.0 mM (■———■), 10.0 mM (▲———▲). Lines intersect at $-K_i$ of approx. 80 µM.

for the mitochondrial and supernatant isozymes of malate dehydrogenase, respectively, Figs. 5 and 6 are classical Dixon plots for the bilirubin inhibition of cytosolic aspartate aminotransferase with

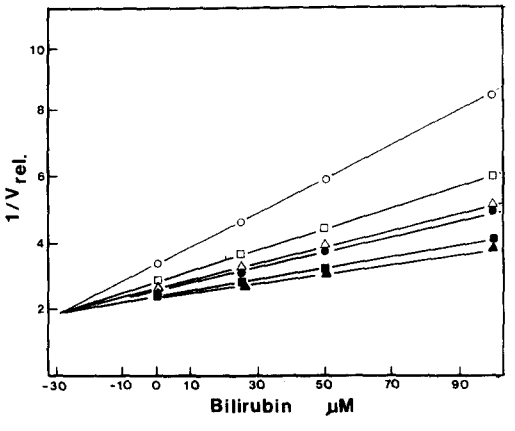

Fig. 6. Inhibition of cytosolic (supernatant) aspartate aminotransferase by bilirubin. Plot of inverse relative reaction velocity vs. micromolar concentration of bilirubin at various concentrations of the substrate 2-oxoglutarate. The concentration of aspartate was constant at 2.5 mM. The concentrations of 2-oxoglutarate are as follows: 0.083 mM (○———○), 0.17 mM (□———□), 0.25 mM (△———△), 0.33 mM (●———●), 0.57 mM (■———■), 1.0 mM (▲———▲). Lines intersect at $-K_i$ of approx. 30 µM.

TABLE I

BILIRUBIN INHIBITION CONSTANTS FOR SELECTED DEHYDROGENASES AND AMINOTRANSFERASES

m-Malate dehydrogenase [EC 1.1.1.37]	
NADH varied	K_i = 2 μM
s-Malate dehydrogenase [EC 1.1.1.37]	
NADH varied	K_i = 15 μM
m-Aspartate aminotransferase [EC 2.6.1.1]	
2-oxoglutarate varied	K_i = 30 μM
aspartate varied	K_i = 100 μM
s-Aspartate aminotransferase [EC 2.6.1.1]	
2-oxoglutarate varied	K_i = 30 μM
aspartate varied	K_i = 80 μM
m-3-Hydroxyacyl coenzyme A dehydrogenase [EC 1.1.1.35]	I_{50} > 150 μM
s-Glycerol-2-phosphate dehydrogenase [EC 1.1.1.8]	I_{50} > 150 μM
s-Alanine aminotransferase [EC 2.6.1.2]	I_{50} = 50 μM
m-Glutamate dehydrogenase [EC 1.4.1.3]	I_{50} = 20 μM

respect to the substrates 2-oxoglutarate and aspartate respectively. These plots demonstrate a competitive mode of inhibition with respect to each substrate and moderate inhibition constants. The data for the mitochondrial form of the enzyme are nearly identical in both a qualitative and quantitative manner. The inhibition constants are summarized in Table I.

Discussion

Previously published observations, combined with the results presented in this study, demonstrate that even low concentrations of free bilirubin are detrimental to mitochondrial functions. Bilirubin has been shown to cause inhibition of mitochondrial function at a concentration of only 3 μM [7,8]. It is interesting that this value is nearly identical to the bilirubin inhibition constant of mitochondrial malate dehydrogenase determined in this study. This inhibition of the dehydrogenase does not appear to be non-specific. It has previously been suggested that the inhibition of dehydrogenases by bilirubin is due to non-specific interactions of the inhibitor with the hydrophobic NADH binding pocket in a domain apparently common to all dehydrogenases [22]. Our results are not in agreement with this proposal and appear to demonstrate a specific inhibition of malate dehydrogenase and perhaps glutamate dehydrogenase by bilirubin. Repeated studies in this laboratory demonstrate that cytosolic glycerol-3-phosphate dehydrogenase is not appreciably inhibited by bilirubin at even the highest concentrations obtainable and that L-3-hydroxyacyl coenzyme A dehydrogenase is inhibited by bilirubin at much greater concentrations than the malate dehydrogenase isozymes. As stated earlier, L-3-hydroxyacyl coenzyme A dehydrogenase has been shown to be very similar in structure to malate dehydrogenase [32–34]. Inhibition of mitochondrial malate dehydrogenase can be disruptive of a number of metabolic pathways. Production and extramitochondrial transport of oxaloacetic acid are dependent upon this enzyme. Inhibition of the tricarboxylic acid cycle and lack of transport of NADH can both result in decreased production of ATP.

All of the aminotransferases examined were moderately inhibited by bilirubin and their inhibition values are similar to values reported for other mitochondrial enzymes. The bilirubin concentrations that inhibit the malate-aspartate shuttle enzymes are within the range of human serum concentrations of bilirubin and mild degrees of hyperbilirubinemia. In serum, nearly all of the bilirubin is bound to serum albumin and its partitioning into the cell is dependent upon the binding constant of bilirubin to cellular proteins. Although the concentrations of free bilirubin within most cells are normally low, bilirubin is known to transport into the cell under conditions of hyperbilirubinemia. The competitive inhibition of 2 μM reported in this study for mitochondrial malate dehydrogenase demonstrates a tight binding of bilirubin to this enzyme. Also noteworthy is that this inhibition value for mitochondrial malate dehydrogenase is nearly identical to the 3 μM value reported for the in vitro inhibition of whole intact mitochondria [7,8]. Although the causes of mitochondrial dysfunction by low concentrations of bilirubin are perhaps due to a large number of combined molecular and physical processes, the results presented in this study suggest strongly that at least one of the molecular processes causing mitochondrial impairment by low concentrations of bilirubin is due to the disruption of the

metabolic system responsible for the transport of reducing equivalents across the inner mitochondrial membrane.

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