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BILIRUBIN: A POTENT INHIBITOR OF NAD$^+$-LINKED ISOCITRATE DEHYDROGENASE

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SUMMARY

Bilirubin was found to be a potent inhibitor of NAD$^+$-linked isocitrate dehydrogenase (*threo*-D$_s$-isocitrate:NAD$^+$ oxidoreductase (decarboxylating), EC 1.1.1.41). It inhibited the enzyme by decreasing the affinity of enzyme for isocitrate, while the maximum reaction velocity was not affected. Bilirubin (less than 10^{-6} M) showed a detectable inhibition. On the other hand, neither the mitochondrial nor the cytoplasmic NADP$^+$ enzymes (*threo*-D$_s$-isocitrate:NADP$^+$ oxidoreductase ((decarboxylating), EC 1.1.1.42) were affected by bilirubin.

INTRODUCTION

Bilirubin was shown to: (1) act as an uncoupler of oxidative phosphorylation[1–4], (2) alter the permeability of mitochondrial membranes by binding to phospholipid[3,5,6], (3) affect electron transfer by inhibiting NADH oxidase and succinate oxidase[7], and furthermore, (4) inhibit mitochondrial enzymes such as glutamate dehydrogenase (EC 1.4.1.2)[8] and malate dehydrogenase (EC 1.1.1.37)[4,9] as well as the particulate NADH dehydrogenase[4]. In spite of these extensive studies, the mechanism of bilirubin toxicity is poorly understood and to date, no evidence of a brain selective toxicity has been presented.

The oxidation of isocitrate to α-ketoglutarate is of special interest because it is usually the rate-limiting reaction of the tricarboxylic acid cycle[10]. Most mammalian tissues contain two types of isocitrate dehydrogenase; one specifically using NAD$^+$ as coenzyme (*threo*-D$_s$-isocitrate:NAD$^+$ oxidoreductase ((decarboxylating), EC 1.1.1.41) and the other NADP$^+$ (*threo*-D$_s$-isocitrate:NADP$^+$ oxidoreductase ((decarboxylating), EC 1.1.1.42). The overall reaction catalyzed by the two types of isocitrate dehydrogenases are identical. Both the NAD$^+$-linked and NADP$^+$-linked isocitrate dehydrogenases have been found in mitochondria of many higher animal tissues, but the former is found only in mitochondria whereas the latter is found both in mitochondria and in the extramitochondrial cytoplasm. The enzymes have been

shown to differ in their molecular weights, as well as in their response to metabolic regulation. In general, the activity of the NAD^+-linked enzyme fluctuates with the concentration of nucleotides[10–13].

In this paper, we describe the inhibitory action of bilirubin on rat brain isocitrate dehydrogenases. Bilirubin is an effective inhibitor of NAD^+-linked isocitrate dehydrogenase, while there is no effect on $NADP^+$-linked enzymes.

MATERIALS AND METHODS

Chemicals

NAD^+, $NADP^+$ and ADP were obtained from Boehringer. Bilirubin was the product of Merck. Trisodium *threo*-D_S-L_S-isocitrate was obtained from Sigma. The term "isocitrate" in this paper always refers to the concentration of *threo*-D_S-isocitrate even though the mixture of *threo* isomer was actually used.

Enzyme assay

NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase activities were measured by the appearance of NAD(P)H, determined by the increase in absorbance at 340 nm. Initial rates were measured in a Hitachi Model 124 or 356 spectrophotometer at room temperature. The reaction mixture for NAD^+ enzyme contained 33 mM Tris–HCl buffer at pH 7.5, 1.33 mM $MnCl_2$, 0.67 mM ADP, 0.33 mM NAD^+, 1.67 mM isocitrate, water and enzyme, in a final volume of 3.0 ml. The $NADP^+$ enzyme was assayed in the reaction mixture which contained 33 mM Tris–HCl buffer at pH 7.5, 1.33 mM $MnCl_2$, 0.05 mM NADP, 0.83 mM isocitrate and 0.33 mM EDTA in a final volume of 3.0 ml. Measurements were made in cuvettes of 1 cm path length and the reaction was started by the addition of enzyme. Deviations from these conditions are indicated in the following text and figures. One unit is the amount of enzyme activity which causes a change of 0.01 in absorbance per min at 340 nm in a volume of 3.0 ml. Specific activity is defined as units per mg of protein. Protein was determined by the method of Warburg and Christian. All kinetic experiments were done with enzyme preparations purified to a specific activity of 600 to 700 units per mg.

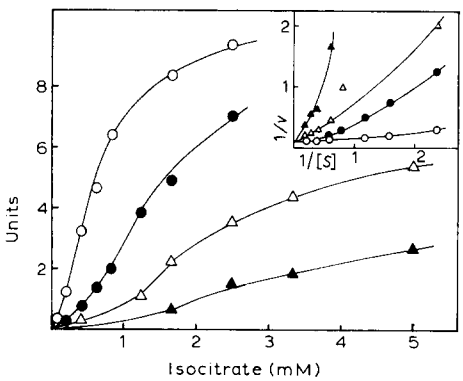
Enzyme purification

NAD^+-linked and the mitochondrial $NADP^+$-linked isocitrate dehydrogenases were purified from rat brain using the crude mitochondrial fraction obtained according to Ozawa *et al.*[14], as a starting material. To solubilize and stabilize the enzymes an equal volume of a solution containing 10 mM potassium phosphate, 10% glycerol, 5 mM $MnCl_2$, 0.1 saturated $(NH_4)_2SO_4$ and 1% Triton X-100 at pH 7.2 was added to the mitochondrial suspension. After centrifugation at 100 000 × *g* for 1 h, the supernatant was fractionated with solid $(NH_4)_2SO_4$. The precipitate obtained from 0.3 to 0.6 saturated with salt was further fractionated with $(NH_4)_2SO_4$ (0.3–0.4 and 0.4–0.5 saturations for the NAD^+ and $NADP^+$ enzymes, respectively). The residue was dissolved in 0.1 saturated $(NH_4)_2SO_4$ and the solution was applied to a Sepharose 6B column (2.6 cm × 60 cm) equilibrated with 0.2 saturated $(NH_4)_2SO_4$ buffer containing 5 mM potassium phosphate, pH 7.2 and 0.01% β-mercaptoethanol. Fractions with enzyme activity were combined and brought to 0.5 saturated with

solid $(NH_4)_2SO_4$. The precipitate was placed in a minimal volume of 0.1 saturated $(NH_4)_2SO_4$ buffer. The cytoplasmic $NADP^+$ enzyme was also partially purified from 100 000 × *g* supernatant of 0.35 M mannitol homogenate with $(NH_4)_2SO_4$ fractionation and gel filtration.

RESULTS AND DISCUSSION

The effects of bilirubin on rat brain isocitrate dehydrogenase as a function of isocitrate concentration at pH 7.5 are shown in Fig. 1. In the absence of ADP the enzyme showed a cooperative effect towards isocitrate. This is readily seen in the insets of Fig. 1 where the data are replotted in terms of $1/v$ against $1/s$. The deviation from linearity showed that the enzyme did not obey Michaelis–Menten kinetics in the absence of ADP. In agreement with the results of others[10–13], inclusion of ADP



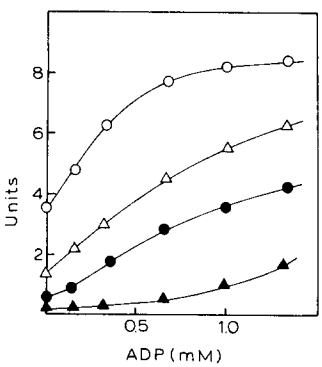


Fig. 1. Effect of isocitrate concentration on the reaction rate of NAD^+-linked isocitrate dehydrogenase activity in the absence or in the presence of ADP or bilirubin. The reaction mixture contained 33 mM Tris–HCl buffer at pH 7.5, 1.33 mM $MnCl_2$, 0.33 mM NAD^+ and different concentrations of isocitrate as indicated in the figure. The activities were determined in the absence (△—△) or presence (○—○) of 1.33 mM ADP and then the effects of 3.5 μM bilirubin were examined without (▲—▲) or with (●—●) 1.33 mM ADP. Inset: Double-reciprocal plots of initial velocity against isocitrate in the absence or presence of bilirubin and ADP.

Fig. 2. Effect of ADP concentration on the reaction rate of NAD^+-linked isocitrate dehydrogenase activity in the absence or presence of 3.5 μM bilirubin. The reaction mixture contained 33 mM Tris–HCl buffer at pH 7.5, 1.33 mM $MnCl_2$, 0.33 mM NAD^+, and different concentration of ADP as indicated in the figure. The concentrations of isocitrate and bilirubin were as follows: ○—○, 1.67 mM isocitrate and no bilirubin; ●—●, 1.67 mM isocitrate and 3.5 μM bilirubin; △—△, 0.83 mM isocitrate and no bilirubin; ▲—▲, 0.83 mM isocitrate and 3.5 μM bilirubin.

in the reaction mixtures increased the apparent affinity of the enzyme for isocitrate. Without ADP the saturation curve was slightly sigmoidal whereby an apparent K_m of 5 mM was obtained. With 1.33 mM ADP the kinetics shifted to near hyperbolic and an apparent K_m of 0.7 mM. It is clear from Fig. 1 that bilirubin affects only the apparent affinity of the enzyme for isocitrate and the V is unaffected by this modifier. Thus, bilirubin inhibits the NAD^+-linked isocitrate dehydrogenase at the lower isocitrate concentrations and the inhibition is overcome with increasing concentration of isocitrate. The overall result is a shifting of the activity curve to the right with bilirubin and a corresponding increase in the sigmoidicity of the curve.

The combined effects of ADP and bilirubin on isocitrate dehydrogenase are

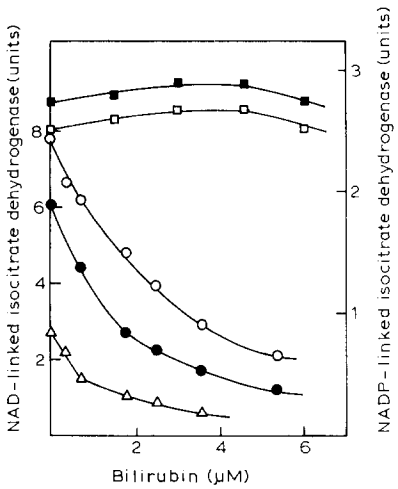


Fig. 3. Effect of bilirubin concentration on the reaction rates of NAD^+-linked isocitrate dehydrogenase and the mitochondrial and cytoplasmic $NADP^+$-linked isocitrate dehydrogenases. The NAD^+-linked isocitrate dehydrogenase activities were determined in the presence of different concentrations of bilirubin and fixed concentrations of isocitrate and ADP (○—○, 1.67 mM isocitrate and 0.67 mM ADP; ●—●, 1.67 mM isocitrate and 0.33 mM ADP; △—△, 2.5 mM isocitrate and no ADP). The $NADP^+$-linked isocitrate dehydrogenase activities were determined in the presence of 3.3 μM isocitrate, 5 μM NADP and different concentration of bilirubin. The partially purified mitochondrial (□—□) and cytoplasmic (■—■) $NADP^+$-linked isocitrate dehydrogenases were used.

shown in terms of v plotted against ADP concentration (Fig. 2). In these experiments, the isocitrate concentration was held constant at 1.67 or 0.83 mM, while ADP concentration was varied. In the absence of bilirubin such plots were hyperbolic. By the addition of bilirubin the plots gradually became sigmoidal. As depicted in Fig. 2, the extent of bilirubin inhibition was more predominant at a lower concentration of isocitrate.

We have also studied the effect of various bilirubin concentrations on the activity of brain NAD^+-linked isocitrate dehydrogenase. A part of these results are shown in Fig. 3. Bilirubin caused powerful inhibition of the enzyme in Tris–HCl buffer at pH 7.5. In these experimental conditions, bilirubin as low as 0.35 μM was found to produce detectable inhibition. The bilirubin concentrations which caused 50% inhibition were 2.5 μM, 1.5 μM and 1 μM with 1.67 mM isocitrate and 0.67 mM ADP, with 1.67 mM isocitrate and 0.33 mM ADP, and with 2.5 mM isocitrate, respectively.

All these finding are consistent with the idea that bilirubin either competes for the isocitrate binding site or occupies a separate allosteric site[15]. When either by increasing the isocitrate or ADP concentration, the enzyme is changed to the active form, the affinity of the enzyme for isocitrate is increased and the inhibitory effect of bilirubin is reduced.

To test whether the inhibitory action of bilirubin is specific for NAD^+-linked isocitrate dehydrogenase, the $NADP^+$-linked enzymes were also purified from the rat brain mitochondrial and cytoplasmic fractions and the effect of bilirubin was examined (Fig. 3). In these experiments, the rate-limiting amounts of isocitrate (3.3 μM) and NADP (5 μM) were used to readily see the effect of bilirubin. Although

the NAD^+ enzyme was strongly inhibited, neither the mitochondrial nor the cytoplasmic $NADP^+$ enzymes were significantly affected up to 6 μM of bilirubin (Fig. 3).

The mode of action of bilirubin has many features[1-9]. However, little is known of the pathogenesis of kernicterus and specific effects on brain tissue in this disease. The difference of bilirubin effects between the NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenases described here is interesting. To test further whether bilirubin inhibition of NAD^+-linked isocitrate dehydrogenase is specific to the brain enzyme, the kidney NAD^+-enzyme was partially purified from the mitochondrial fraction and the effect of bilirubin on enzyme activity was also tested. In these experiments, bilirubin was observed to inhibit kidney enzyme (unpublished), indicating that the inhibition is not specific to the brain enzyme. However, as observed by other workers[11,13] and also confirmed in our laboratory, the total isocitrate dehydrogenase activity in mitochondria is lower in the brain than in other tissues, while the contribution of NAD^+ enzyme in mitochondria is much higher in brain tissue. Thus, the brain-specific effects of bilirubin could be explained on the basis of a specific distribution of NAD^+-linked isocitrate dehydrogenase in the brain mitochondria. The NAD^+-linked isocitrate dehydrogenase reaction is more sensitive to bilirubin than other dehydrogenases such as glutamate and malate dehydrogenases. According to the report of Noir *et al.*[4], the bilirubin concentrations that decreased activity to one-half were 7 μM and 40 μM for the crude preparations of rat liver mitochondrial glutamate- and malate dehydrogenases, respectively and were 28 μM and 12 μM for crystalline bovine liver glutamate dehydrogenase and pig heart malate dehydrogenase, respectively, in their experimental conditions. These values are much higher than those obtained in our experiments for NAD^+-linked isocitrate dehydrogenase. Furthermore, the fact that bilirubin inhibition is more predominant at a lower concentration of isocitrate (Figs 1 and 2) is of interest considering the low level of isocitrate *in vivo*[16]. Although the significance *in vivo* of the bilirubin inhibition of NAD^+-linked isocitrate dehydrogenase is not known, the very low concentration of bilirubin necessary to alter the activity *in vitro* suggests that the phenomenon could be of significance in the pathogenesis of bilirubin encephalopathy.

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