BILIRUBIN: A MULTI-SITE INHIBITOR OF MITOCHONDRIAL RESPIRATION

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1. Introduction

Unconjugated bilirubin is a toxic substance that interferes with the normal cell functions [1–4]. Despite several studies [1–8] that indicate an effect on the terminal oxidative metabolism the mechanism of bilirubin action is not yet known. The pigment, i) affects electron transfer [2], by inhibiting NADH-oxidase and succinate oxidase, ii) acts as a typical uncoupler [5], and iii) alters the permeability of mitochondrial membranes, inducing swelling [6, 7]. Mustafá and King [8] showed that bilirubin binds to mitochondrial phospholipids, a property that might lead to a general explanation of bilirubin action on mitochondria. In fact, by binding to mitochondrial lipids, bilirubin would produce an alteration of mitochondrial membranes, and consequently, inhibition of electron tranfer, uncoupling and swelling.

The observations described here demonstrate that besides the above mentioned effects, bilirubin acts as an effective inhibitor of mitochondrial NAD-linked dehydrogenases, namely glutamate dehydrogenase (EC 1.4.1.2) and malate dehydrogenase (EC 1.1.1.37). These latter inhibitions are significant in order to understand the effect of bilirubin on mitochondrial respiration. Moreover, additional information on the site of action of bilirubin on the respiratory chain is presented.

2. Material and methods

Rat liver mitochondria were isolated in a medium containing 0.25 M sucrose, 0.2 mM EGTA and 5 mM Tris-HCl (pH 7.4) according to Schneider's method [9]. Submitochondrial particles from rat liver were obtained by sonication in a MSE sonifier model 500 W (Measuring & Scientific Equip. Ltd., London) at an output of 0.7 mA according to the method of Kielley and Bronk [10]. Mitochondrial glutamate- and malate-dehydrogenase were obtained by osmotic shock [11] from mitochondria incubated in 3 mM Tris-HCl (pH 7.4) for 10 min at 0°. After centrifugation at 105 000 *g* for 10 min, the supernatant was separated and the operation repeated with the pellet. Both supernatants were pooled and used as the source of glutamate- and malate-dehydrogenases. Bovine liver glutamate dehydrogenase, type I, and malate dehydrogenase from pig heart were purchased from Sigma Chemical Company.

Mitochondrial oxygen consumption was determined polarographically at 30° with a model K Oxygraph (Gilson Medical Electronics). The reaction medium contained 200 mM sucrose, 35 mM KCl, 6 mM $MgCl_2$, 6 mM Na_2HPO_4–KH_2PO_4, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4). Pyridine nucleotide fluorescence

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was measured with the fluorescence attachment of the Aminco-Chance spectrophotometer (American Instruments Co.) equipped with a primary filter Chance OX-1 with transmittance maximum at 366 nm and a secondary filter Wratten 2A passing light above 405 nm. Activities of glutamate- and malate-dehydrogenases were measured at 30° by following the decrease of absorbance of NADH at 340 nm. Reaction velocities were calculated from the linear part of the recordings. NADH-dehydrogenase was determined according to Minakami et al. [12]. Protein determinations were carried out by the biuret method [13] in the presence of 0.1% deoxycholate for the mitochondrial suspensions, and by the Warburg and Christian method [14] for the commercial enzymes. Bilirubin (British Drug Houses Ltd.) was added to the reaction mixtures as aliquots of a 4.2 mM stock solution in 0.02 M NaOH. Corresponding amounts of 0.02 M NaOH were added to the control mixtures.

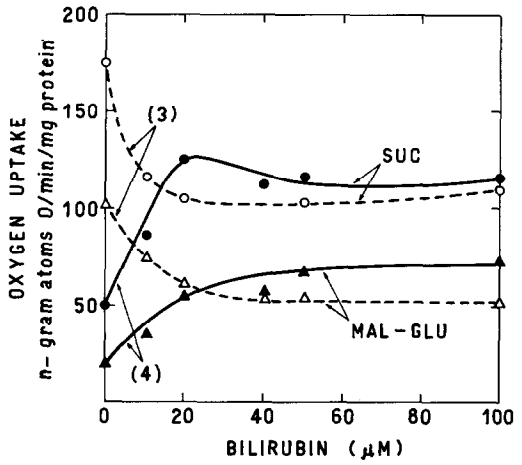

Fig. 1. Effect of bilirubin on the oxygen consumption of rat liver mitochondria. Substrate malate–glutamate (Mal–Glu): 6 mM malate, 6 mM glutamate and 3 mM malonate. Substrate succinate (Suc): 10 mM succinate and 0.2 μM rotenone. State 3: 1 mM ADP, 1.1–1.6 mg mitochondrial protein/ml. Other conditions as described under Methods. In parenthesis, metabolic state.

3. Results

3.1. *Effect of bilirubin on mitochondrial respiration*

The addition of bilirubin to mitochondrial suspensions oxidizing malate–glutamate or succinate in state 3 [15] produced a marked inhibition of the oxygen consumption (fig. 1). The inhibition, similar for both substrates, was maximal, about 50%, at 20–40 μM bilirubin. In contrast, addition of bilirubin to the same mitochondrial suspensions in state 4 [15] produced an increase in the respiration rate (fig. 1). These results along with those earlier reported by others [2, 5, 7] indicate that bilirubin has two effects, namely, inhibition of electron transport and uncoupling of oxidative phosphorylation.

Concerning the effects on electron transport, it was found that bilirubin acted additively with rotenone, a known inhibitor of mitochondrial NADH-dehydrogenase. In the experimental conditions of fig. 1, with malate–glutamate as substrate, 20 μM bilirubin inhibited oxygen consumption by 34%, 30 nM rotenone by 37%, and the addition of both inhibitors by 70%. Similarly, with succinate as substrate, 20 μM bilirubin inhibited oxygen consumption by 36%, 10 ng/ml antimycin by 30%, and the addition of both inhibitors by 60%. These results indicate that bilirubin inhibits electron transport at both the rotenone- and the antimycin-sensitive sites.

3.2. *Effect of bilirubin on the fluorescence of intramitochondrial pyridine nucleotide*

Malate–glutamate and antimycin were added to a mitochondrial suspension to fully reduce intramitochondrial pyridine nucleotide. Addition of bilirubin produced a quenching of the pyridine nucleotide fluorescence, as shown in fig. 2. This was expected since bilirubin absorbs in the 400–450 nm region producing an "inner filter" effect. The semi-logarithmic plot in fig. 2 shows that the effect of bilirubin as fluorescence quencher was more marked upon intramitochondrial pyridine nucleotide than on a NADH solution. The pigment concentrations required to reduce to one-half the initial fluorescence of NADH solutions were 26–30 μM, and these values were independent of NADH concentration in the range of 10–50 μM. A similar effect upon intramitochondrial pyridine required 8–12 μM bilirubin, and the values depended on the amount of pyridine nucleotide (or mitochondrial protein). Since "bound"-NADH fluores-

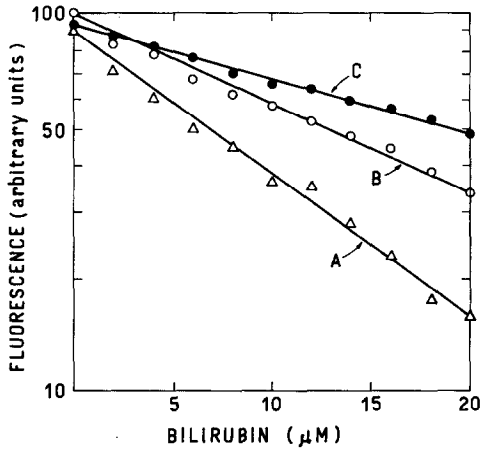

Fig. 2. Quenching by bilirubin of pyridine nucleotide fluorescence. Line A) Fluorescence of intramitochondrial pyridine nucleotide fully reduced in the presence of malate–glutamate (exptl. conditions as in fig. 1) and antimycin 0.1 μg/ml; 1.2 mg mitochondrial protein/ml. Line B) Same, but 0.3 mg mitochondiral protein/ml. Line C) Fluorescence of 10 or 50 μM NADH (similar results were obtained with both concentrations).

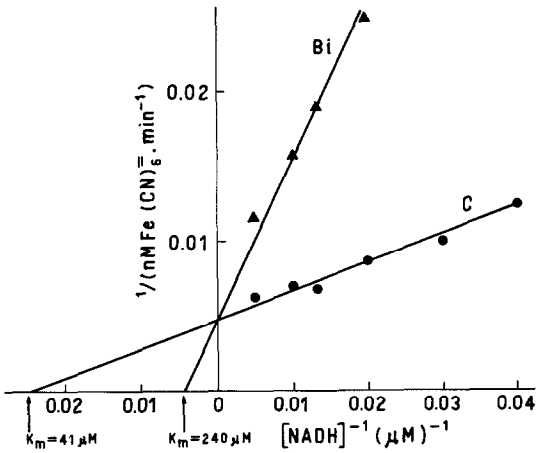

Fig. 3. Lineweaver–Burk plot for the effect of bilirubin on NADH-dehydrogenase. 8 μM bilirubin (Bi), 1.5 mM ferricyanide and 50 mM Na_2HPO_4–KH_2PO_4 (pH 7.4). Submitochondrial particles from rat liver, 0.02 mg/ml. C, control activity.

ces more intensely than "free"-NADH [16] these results might be interpreted as an interference by bilirubin with the binding of NADH to protein. An alternative explanation might be an inhibitory action of bilirubin on malate and/or glutamate dehydrogenases that shifts the equilibrium towards pyridine nucleotide oxidation.

3.3. *Effect of bilirubin on NADH-dehydrogenase*

Bilirubin (8 μM) inhibited 45% of NADH-dehydrogenase activity in submitochondrial particles from rat liver in the presence of 150 μM NADH and 1.5 mM ferricyanide. This inhibitory effect increased with increasing amount of bilirubin. Double reciprocal plots (fig. 3) showed that the inhibition by 8 μM bilirubin was competitive, increasing the apparent K_m for NADH from 41 μM to 240 μM. Since bilirubin is susceptible to oxidation and ferricyanide is an oxidant, a direct reaction of both is to be expected. In the experimental conditions of fig. 3, reduction of ferricyanide was measured as the decrease of absorbance at 420 nm. Appropriate controls, as well as the measured inhibition of the rate of ferricyanide reduction showed that bilirubin actually affected NADH-dehydrogenase activity.

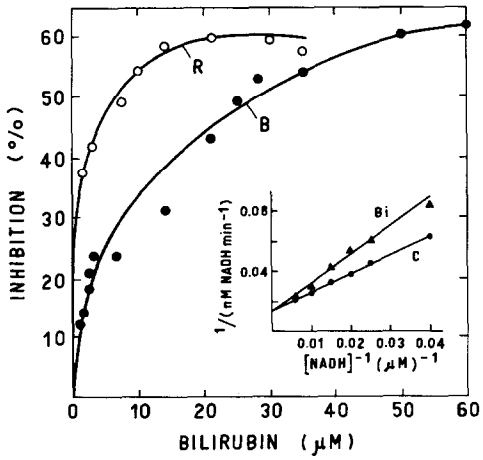

Fig. 4. Inhibition of glutamate dehydrogenase by bilirubin. Line B, enzyme from bovine liver, 0.66 μg/ml, specific activity: 43 μmoles NADH/min/mg. Line R, enzyme from rat liver mitochondria, 92 μg total protein/ml, specific activity: 0.17 μmoles NADH/min/mg. The reaction medium consisted of 2 mM α-ketoglutarate, 100 mM NH_4Cl, 100 μM NAD, 1 mM EDTA and 25 mM Tris-HCl (pH 8.1). *Insert:* Lineweaver–Burk plot for the effect of bilirubin on glutamate dehydrogenase. Reaction medium as above, except for 50 mM Na_2HPO_4–KH_2PO_4 (pH 7.4) that was used instead of Tris-HCl and the variable concentration of NADH. 22 μM bilirubin (Bi) and 1.2 μg/ml enzyme, C, control activity.

3.4. *Effect of bilirubin on glutamate- and malate-dehydrogenase*

Bilirubin inhibited crystalline glutamate dehydrogenase activity from bovine liver and also a crude preparation of enzyme obtained from rat liver mitochondria. The concentrations that decreased activity to one-half (I_{50}) were 7 μM and 28 μM for the rat liver and the bovine liver enzymes, respectively (fig. 4). Inhibition was competitive with NADH, and a pigment concentration of 22 μM changed the apparent K_m for the coenzyme from 82 μM to 140 μM (fig. 4, insert). On the other hand, an experiment carried out with the bovine liver enzyme, varying the α-ketoglutarate concentration, showed that the inhibition was non-competitive. The apparent K_i for bilirubin, obtained from a Dixon plot [17] was 28 μM.

Malate dehydrogenase from pig heart and a crude preparation from rat liver mitochondria were both inhibited by the pigment. I_{50} values of 12 μM and 40 μM were obtained, respectively (fig. 5). The inhibition was competitive with NADH: 13 μM bilirubin changed the apparent K_m for the coenzyme from 10 μM to 45 μM (fig. 5, insert).

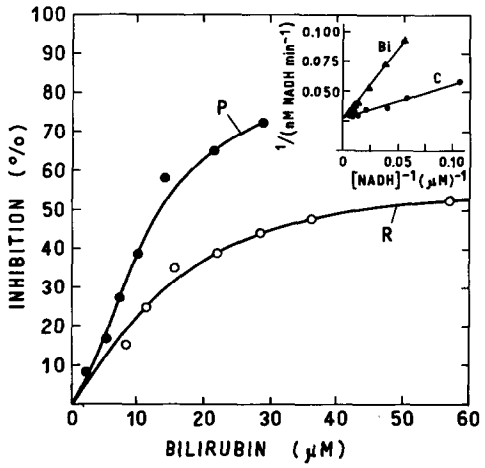

Fig. 5. Effect of bilirubin on malate dehydrogenase. Line P, enzyme from pig heart, 0.04 μg/ml, specific activity: 384 μmoles NADH/min/mg. Line R, enzyme from rat liver mitochondria, 46 μg/ml, specific activity: 0.45 μmoles NADH/min/mg protein. Reaction medium: 100 μM NADH, 50 μM oxaloacetate, 25 mM Tris-HCl (pH 7.4). *Insert:* Lineweaver–Burk plot for the effect of bilirubin on pig heart malate dehydrogenase. Exptl. conditions as above except for 0.03 μg/ml enzyme, 50 mM $Na_2HPO_4-KH_2PO_4$ instead of Tris-HCl, 13 μM bilirubin (Bi) and variable concentration of NADH. C, control activity.

4. Discussion

Previous observations, combined with our own results, demonstrate that bilirubin behaves as a multisite inhibitor of mitochondiral respiration. It acts as an uncoupler of oxidative phosphorylation ([2, 5, 7] and fig. 1), and independently of the mechanism of uncoupling, bilirubin affects the structural state of mitochondira [7, 18]. In fact, uncoupling possibly results from alteration of membrane permeability and subsequent induction of ion transport [7, 8]. The pigment also inhibits electron transport at both the rotenone- and the antimycin-sensitive sites (see Results). Furthermore, bilirubin inhibits soluble mitochondrial enzymes such as glutamate dehydrogenase ([19] and fig. 4) and malate dehydrogenase (fig. 5), as well as the particulate NADH-dehydrogenase (fig. 3). The bilirubin concentration that inhibits these dehydrogenases is in the range of the pigment concentration that affects mitochondrial respiration. Therefore, the inhibition of the dehydrogenses may be considered as a significant contribution to the overall effect of bilirubin on mitochondrial functions. In all cases bilirubin competes with the reduced coenzyme (figs. 3–5), suggesting that the pyrrol groups of the pigment molecule would interact at the active site with the same groups as do the pyrrol of the adenine moiety and the pyridinium group of NADH. Moreover, the hydrophobicity of the tetrapyrrol would fit in with the similar characteristics of the NADH-binding site.

The bilirubin concentration that severely impair mitochondrial function *in vitro*, namely 10–30 μM, corresponds to values of 0.6–1.7 mg/100 ml which are within the normal human serum concentration of the pigment and relatively mild degrees of hyperbilirubinemia. Studies *in vivo* with kernicterus Gunn rats showed a decreased level of ATP in hyperpigmented and functionally damaged areas of brain [3] which would reveal mitochondrial damage. However, no diminution of intrahepatic ATP level could be detected in icteric animals [20]. On these grounds it is diffucult to assess the physiological implications of our results. In fact, the tight binding of bilirubin to serum albumin [21] may prevent the diffusion of bilirubin into the

cells and the mitochondria; on the other hand, a long time exposure to high pigment levels might cause mitochondrial damage because bilirubin could be adsorbed by cells even at bilirubin: albumin ratios within the normal binding capacity of human serum albumin [4].

Acknowledgements

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