ON THE ENERGY-DEPENDENT BILIRUBIN-INDUCED MITOCHONDRIAL SWELLING

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Received October 27, 1967

According to an early report, bilirubin is a good uncoupler with an apparent K value of about 20 μM in the phosphorylation from the oxidation of glutamate (1). The bile pigment also disturbs other respiratory enzyme systems but at much higher concentrations (2). In this note, we report the effect of bilirubin on the swelling of mitochondria and some collateral observations.

As shown in Fig. 1, both the rate and amplitude of the swelling were dependent upon the concentration of bilirubin. However, the rate decreased with the increase of bilirubin concentration when the latter was higher than 20 μM (see below for explanation). The K value of bilirubin for the overall swelling was found to be about 2.5 μM (equivalent to less than 0.15 mg per 100 ml). Biliverdin tested at 20 μM and 50 μM did not show any effect.

An energy source was essential for the swelling. The process did not take place if the energy derived from either electron transport or exogenous ATP was blocked as depicted in Fig. 2. It might be noted from the figure that bilirubin alone, at a concentration as high as 10 μM, did not induce any swelling. Upon addition of β-hydroxybutyrate the swelling took place rapidly. But the process was completely inhibited by rotenone, a known potent

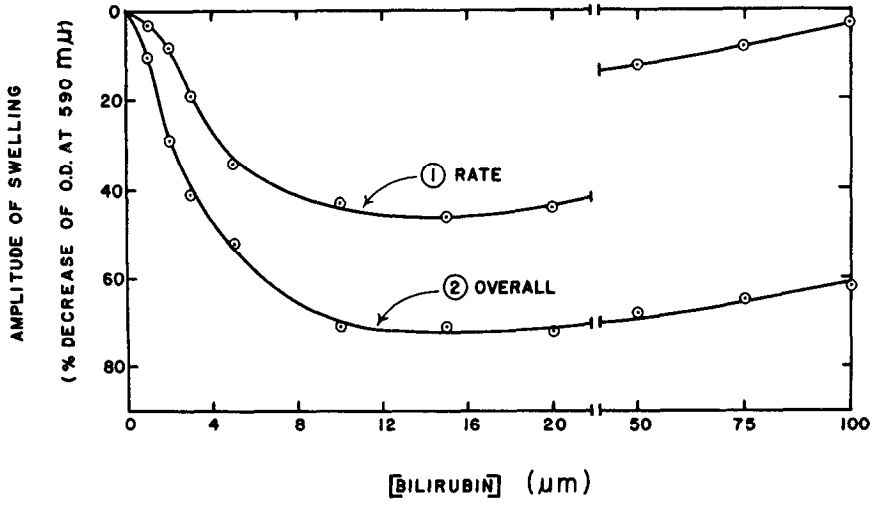

Fig. 1. Effect of concentration of bilirubin on the swelling of hepatic mitochondria. The system contained 100 mM sucrose, 10 mM mannitol, 8 mM $MgCl_2$, 5 mM potassium phosphate, 5 mM Tris-Chloride, 0.8 mg per ml (in terms of protein) of rat liver mitochondria and 10 mM β-hydroxybutyrate; pH, 7.5. The ordinate is the amplitude of the swelling expressed in the percent of decrease of absorbancy at 590 mμ. The rate is expressed as the percent decrease for the first minute.

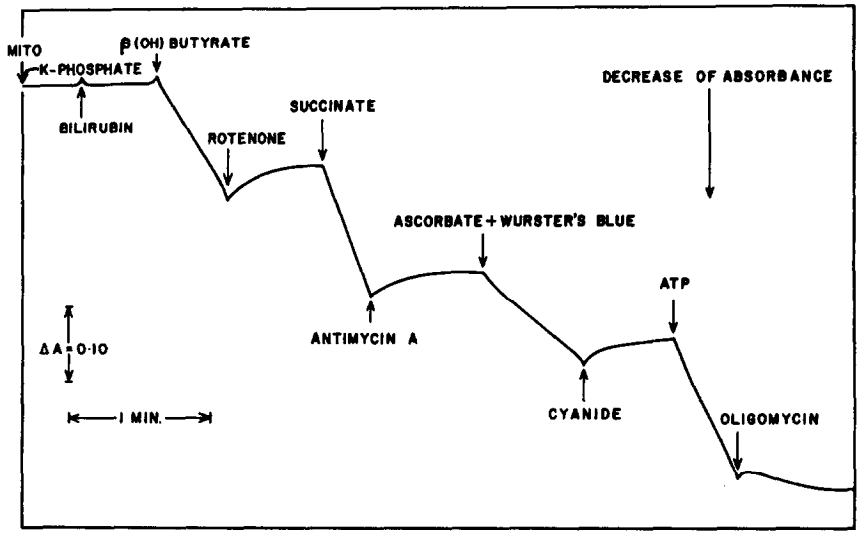

Fig. 2. Demonstration of energy requirement for the swelling of hepatic mitochondria. The basal mixture was the same as that described in Fig. 1 except no substrate was present at the beginning. The additions as shown were 10 μM bilirubin, 10 mM β-hydroxybutyrate, 0.6 μM rotenone, 10 mM succinate, 0.5 μg antimycin A per mg protein, 5 mM ascorbate + 100 μM Wurster's blue, 0.1 mM cyanide, 2.5 mM ATP and finally 1 μg oligomycin per mg protein present.

respiratory poison for the oxidation of NADH linked substrates. Further addition of succinate, whose oxidation is not inhibited by rotenone, elicited the process to resume. Antimycin A, which inhibits the oxidations of both β-hydroxybutyrate and succinate, also inhibited the swelling.

Recently, it has been shown that ascorbate-Wurster's blue system can by-pass the inhibitions of rotenone and antimycin leaving only the terminal site of cytochromes c and oxidase functional, yielding an equivalent of P/O ratio of unity (3, 4). As shown in Fig. 2, upon addition of ascorbate-Wurster's blue, the swelling process resumed. When the terminal system was completely inhibited by cyanide, the swelling also ceased. Under these conditions, the introduction of exogenous ATP recovered the swelling reaction. Not until the addition of oligomycin, a strong inhibitor for ATP-linked energy-transfer reactions, did the swelling process stop.

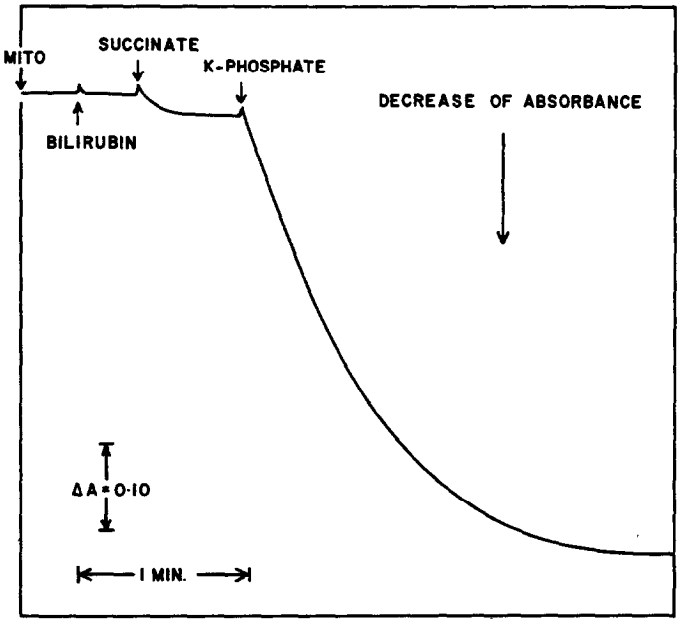

Fig. 3. Requirement of ions for the swelling of hepatic mitochondria. The basal mixture was the same as that described in Fig. 2, except no potassium phosphate was present at the beginning. The additions were 10 μM bilirubin, 10 mM succinate and 10 mM potassium phosphate, pH 7.5.

This sort of the energy-dependent bilirubin-induced swelling of heptic (as well as cerebral) mitochondria was demonstrated in various designs of experiments and Fig. 2 is only a protocol. Other respiratory inhibitors and uncoupler so far tested showed a clear cut (100%) inhibition of the swelling. The reagents used were pentachlorophenol, Seconal, hydroxyquinoline-N-oxide, sulfide and azide. Bovine serum albumin, which can "detoxify" bilirubin by conjugation (cf. 5), even at as low as 0.1 mg per ml, inhibited the swelling induced by 10 μM bilirubin.

The swelling evidently resulted from the ion and water movements involved in the process. A cation and anion source such as potassium phosphate (as shown in Fig. 3) or sodium acetate (data not shown), other than sodium succinate or β-hydroxybutyrate,* was essential for the process. Likewise, Mg^{++} ions were also required, in its absence the swelling became oscillatory in nature. In this respect, the mechanism of bilirubin action differs from that of gramicidin (6), parathyroid hormone (7), or EDTA (8) in which Mg^{++} exerts some kind of a protective action against the swelling. The details of ion transfer (both the oscillatory and "steady state" aspects) will be reported elsewhere.

We also found that bilirubin exerted a biphasic response toward the respiration as shown in Fig. 4. The respiration measured as oxygen consumption increased with the increase of bilirubin concentrations up to about 20 μM; further increase decreased the respiratory rate. The latter phenomenon is evidently responsible for the decreased rate of swelling at high

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*The substrate, sodium β-hydroxybutyrate or sodium succinate present in the Mg^{++}-containing medium was not effective for swelling due to slow movement of the conjugate anions, although these anions could enter the mitochondrion at a rate sufficient to support the respiration. Km values for these substrates under the conditions tested are rather small.

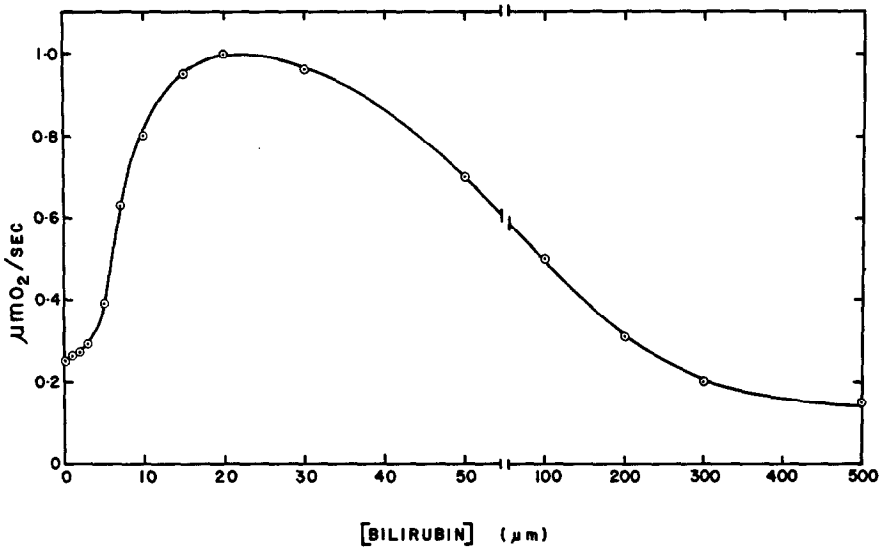

Fig. 4. Effect of bilirubin on the respiration of hepatic mitochondria. The basal mixture was the same as Fig. 1.

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concentrations of bilirubin as illustrated in Fig. 1.

The effect of bilirubin was more sensitive toward the respiratory control than to ADP/O ratio. On the effect of the respiratory control, K values for bilirubin were found to be approximately 4 μM in both the β-hydroxybutyrate and the succinate system. Under the same experimental conditions, the equivalent K values on the effect of the decrease of ADP/O ratio were about 12 μM.

In summary, the effects of bilirubin on mitochondrial reactions were four-fold: viz. respiration, the respiratory control index, ADP/O ratio and the energy dependent swelling were all affected. The swelling process may proceed through the following sequence of events. Bilirubin may specifically conjugate with certain components of the mitochondrial membrane thus altering membrane** and permitting ions and exogenous ATP (whenever added) to enter (cf. Fig. 2). The alteration of membrane may

then be followed by a transfer of both cations and anions as well as water from the medium into the mitochondrion. Eventually, the swelling takes place. Thus, swelling is a manifestation of energy-dependent ion movements through the altered membrane. The absolute requirement of energy explains the previous failure (e.g. 9) to demonstrate this effect of bilirubin on mitochondrial swelling.

**It is a well known fact that bilirubin, in addition to its lipophilic properties, can combine with a number of proteins.

Acknowledgements -- This work was supported by grants from the National Science Foundation, the U.S. Public Health Service, the American Heart Association and the Life Insurance Medical Research Fund. M. L. C. is an NIH Career Development Awardee.

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