Binding of Bilirubin with Lipid

A POSSIBLE MECHANISM OF ITS TOXIC REACTIONS IN MITOCHONDRIA*

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SUMMARY

Binding of bilirubin with mitochondria, mitochondrial membranes, various cells, and cellular fragments resulted in a hypochromicity with a red shift of the absorption spectrum of bilirubin. It was found that the mitochondrial lipids rather than the proteins were involved in the binding. Isolated mitochondrial lipid and lipids from various other sources caused similar spectral changes of the bilirubin absorbance. Exogenous lipid could prevent the toxic effect of bilirubin on well-defined mitochondrial reactions as did serum albumin.

Washing of bilirubin-treated mitochondria with albumin restored their normal function to a considerable extent. These findings suggest that the binding of bilirubin with lipid alters the characteristics of mitochondrial membranes so that membrane-linked functions are impaired. In view of the lipophilic nature of bilirubin, this explanation might be considered as a mechanism of toxicity of bilirubin for cells in general.

A number of studies in vitro have shown that unconjugated bilirubin is toxic to such fundamental processes as cellular respiration (2-7), oxidative phosphorylation (1, 7-12), electron transport (1, 7-13), permeability, and the structural state of mitochondria (1, 11, 12, 14). The characteristic effects that we have observed of bilirubin on mitochondrial reactions include stimulation of respiration at low concentrations of bilirubin but inhibition at high concentrations, abolition of respiratory control, uncoupling of oxidative phosphorylation, and swelling of mitochondria (1, 11). All of these reactions are actually manifestations, in greater or lesser extent, of mitochondrial membranes.

Serum albumin is an effective agent for detoxifying bilirubin (16-18). The detoxification is due to a remarkably tight binding of this bile pigment with albumin. Clinically, albumin in conjunction with exchange transfusion has been used for the treatment of hyperbilirubinemia in newborn infants (see for example, References 19 and 20). In vitro, a little over 0.5 mg of bovine serum albumin per ml is sufficient to prevent the adverse effects exerted by 10 μM bilirubin on well-defined mitochondrial reactions (1, 11).

Despite diligent work in many laboratories for a number of years, the mechanism of bilirubin toxicity is not known. Recently we found that bilirubin binds with mitochondria. The component in mitochondria responsible for the binding is not protein but lipid. Exogenous lipids can alleviate the adverse effects of bilirubin. Results presented in this communication suggest that the binding affects the membrane-linked functions of mitochondria in particular, and perhaps also those of intact cells in general. Serum albumin can reverse the binding of bilirubin with lipids by forming a characteristic bilirubin-albumin complex.

EXPERIMENTAL PROCEDURE

Preparations—Rat liver mitochondria were isolated in a medium containing 250 mM sucrose, 25 mM mannitol, and 0.1 mM EDTA, pH 7.5, and bovine heart mitochondria in the same medium but containing in addition 20 mM Tris-chloride according to procedures described previously (1, 21). The Keilin-Hartree preparation from bovine heart was obtained according to the original method (22) as adapted in this laboratory (23). The outer and inner membranes of rat liver mitochondria were prepared and purified following the procedure of Parsons et al. (24-26).

Other Materials—Bilirubin solution was prepared by quickly dissolving 8.8 mg of the crystalline substance in 0.2 ml of 0.1 N KOH, diluting it to a final volume of 5 ml with water, and adjusting the pH to about 9. The solution was kept in the dark and used not more than 6 hours after preparation. Lipids were isolated from rat liver mitochondria and ascites tumor cells by extracting with a chloroform-methanol (2:1) mixture (cf. References 27 and 28). Lipid suspensions were prepared in 50 mM phosphate plus 0.1 mM EDTA, pH 7.5, by sonic oscillation in an MSE 100-watt ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London) for 30 min, and the lipid micelles were obtained in the supernatant by centrifugation at 105,000 × g for an hour (cf. Reference 29). The lipid content was determined gravimetrically.

Lipid-deficient heart mitochondria (about 80% lipid-depleted) were prepared by extraction of lipid with 10% water in acetone according to the method of Fleischer and Fleischer (28). Lipid-extracted liver mitochondria and the heart muscle preparation
were obtained by exhaustive extraction with a chloroform-methanol (2:1) mixture.

Erythrocyte membrane was kindly supplied by Dr. D. J. Hanahan, tissue culture cells by Dr. M. L. Cowger, and ascites tumor cells by Dr. C. P. Lee and Dr. T. Galiotti. Samples of bilirubin were purchased from Sigma. Other chemicals were of the purest grade commercially available. Deionized distilled water was used after redistillation in all glass apparatus.

Methods—All assays were conducted at room temperature, about 24°C, in systems as detailed in the legends to the figures and tables. Protein was determined by the biuret method (30).

Measurements of oxygen uptake and oxidative phosphorylation were performed polarographically in a Gilson Medical Electronics (Middleton, Wisconsin) oxygraph (model K) with a Clarke electrode. Swelling was measured by recording the decrease of absorbance at 590 nm in a Cary model 14 spectrophotometer (1).

RESULTS

Free bilirubin in solution showed an absorption maximum of 440 nm with a millimolar extinction coefficient of about 52. The addition of mitochondria to a bilirubin solution caused a hypochromicity with a red shift as depicted in Fig. 1; a broad peak centered approximately at 450 nm and a shoulder around 492 nm were observed. These spectral changes were not due to any artifact such as turbidity or nonspecific destruction of bilirubin. The amount of mitochondria added corresponded to less than 0.02 absorbance unit. Moreover, the characteristic spectrum of the bilirubin-albumin complex emerged upon addition of bovine serum albumin to the bilirubin-mitochondrial system, indicating that the bile pigment was not destroyed in the process of binding with mitochondria.

The binding of bilirubin with mitochondria was found to be time-dependent as shown in Fig. 2. The rate of absorbance decrease (i.e., binding) was dependent upon the concentrations of mitochondria as well as bilirubin.

Since a microgram quantity of mitochondria could cause a significant spectral change of bilirubin absorption, it was of importance to determine if any specific functional or structural state of mitochondria was involved in the process. We found no effect whatsoever on the absorption change by addition of respiratory inhibitors such as rotenone, antimycin, or cyanide; or treatment of mitochondria with 0.6 M urea or surface active agents, such as 2% cholate or 2% Triton X-100. These results, therefore, ruled out the possibility that the structural integrity or functional state of mitochondria was required for the spectral change. We also tested the binding of bilirubin with mitochondrial membrane fractions. No difference was observed between outer and inner membranes, although the rate and the extent of absorbance change were dependent upon their lipid contents.

We therefore suspected that mitochondrial lipid might be involved in the process. Experiments, such as summarized in Fig. 3, testified affirmatively to this point. The rate of decrease of bilirubin absorbance at 440 nm in the presence of mitochondrial lipid was similar to that in the presence of whole mitochondria (Fig. 2). Likewise, the absorption spectra were practically identical (Fig. 1 versus Fig. 3). Again, bovine serum albumin was able to restore the characteristic bilirubin-albumin complex from the system containing bilirubin and mitochondrial lipid.

The investigation regarding the binding state of bilirubin was extended to see if this pigment could bind with lipid-deficient cardiac mitochondria and membranes. Addition of lipid-deficient cardiac mitochondria (by aqueous acetone extraction, about 80% of lipid removed) to a bilirubin solution brought about the same characteristic changes in the absorption spectrum as depicted in Fig. 1 or 3. It should be pointed out that even as small an amount of lipid as 1 μg per ml could cause an effective spectral change. Therefore, the lipid that remained in the extracted mitochondria was evidently responsible for the binding.

Fig. 1. Effect of mitochondria on the absorption spectrum of bilirubin. Basal medium contained 50 mM potassium phosphate and 50 mM Tris-chloride, pH 7.5. In addition, Curve A contained 13.2 μM bilirubin; Curve B, 13.2 μM bilirubin and 20 μg (protein) of rat liver mitochondria per ml; Curve C, 13.2 μM bilirubin and 6 μg of bovine serum albumin per ml; Curve D, same as Curve B but also with 6 μg of bovine serum albumin per ml. Appropriate blanks containing mitochondria and bovine serum albumin were used in the reference cell.

Fig. 2. Effect of time on the decrease of A440 of bilirubin in the presence of mitochondria. Basal medium was the same as in Fig. 1; 36 μM bilirubin was used and amounts of mitochondria as shown.
Addition of 17 μM bilirubin abolished phosphorylation and gave high rates of respiration typical of an uncoupled state of mitochondria. The results summarized in Table I and II and Fig. 4 graphs, lipid should be able to alleviate the adverse effects of are representative examples to illustrate the protective actions characteristic absorption.

**Fig. 3. Effect of mitochondrial lipid on the absorption spectrum of bilirubin.** Basal medium was the same as in Fig. 1. In addition, Curve A contained 13.2 μM bilirubin; Curve B, 13.2 μM bilirubin and 15 μg of mitochondrial lipid per ml; Curve C, 13.2 μM bilirubin and 6 mg of bovine serum albumin per ml; Curve D, same as Curve B but also with 6 mg of bovine serum albumin per ml.

observed. As expected, a larger amount (in terms of total weight) of the lipid-deficient preparation was needed to achieve a change of bilirubin absorption spectrum comparable to that obtained in the presence of nonextracted mitochondria. On the other hand, the addition of exhaustively-extracted hepatic mitochondrial or cardiac submitochondrial particles (prepared by thorough extraction of lipid with 2:1 mixture of chloroform-methanol) did not cause any change of the absorption spectrum of bilirubin, indicating that no binding took place when the lipid was evidently completely removed from these preparations.

Does bilirubin bind with specific lipids only? Results from numerous experiments showed that this was not the case. Bilirubin in solution reacted with various cells and cellular components containing lipid, such as ascites tumor cells, ascites cell mitochondria, tissue culture cells, yeast cell fragments, and erythrocyte membrane. Likewise, bilirubin bound to ascites cell lipid, asolectin (soybean lipid), triolein, and commercial vegetable oils. The spectra obtained were virtually the same as those depicted in Figs. 1 and 3. In all cases, bovine serum albumin displaced the lipid from the lipid-bound bilirubin to form a bilirubin-albumin complex as witnessed by the characteristic absorption.

If our reasoning is correct as implied in the preceding paragraphs, lipid should be able to alleviate the adverse effects of bilirubin on those reactions manifested by mitochondrial membranes. The results summarized in Table I and II and Fig. 4 are representative examples to illustrate the protective actions of lipid against bilirubin. Oxidation of β-hydroxybutyrate (cf. Table I) and of succinate (cf. Fig. 4) by mitochondria resulted in ADP:O ratios of about 2.8 and 1.8, respectively. Addition of 17 μM bilirubin abolished phosphorylation and gave high rates of respiration typical of an uncoupled state of mitochondria. In the presence of exogenous mitochondrial lipid, the

### Table I

**Effect of lipids on bilirubin-induced uncoupling of oxidative phosphorylation**

Basal medium contained 100 mM sucrose, 75 mM mannitol, 8 mM Tris-chloride, 5 mM potassium phosphate, 5 mM MgCl₂, and 1.5 mg (in terms of protein) of rat liver mitochondria per ml, pH 7.5. Other additions as indicated were 10 mM β-hydroxybutyrate, 150 μM ADP, 17 μM bilirubin, 7.5 μg of mitochondrial lipid per ml, 7.7 μg of asolectin per ml, 25 μg of ascites cell lipid per ml, 50 μg of triolein per ml, 50 μg of commercial vegetable oil per ml, 50 μg of linoleic acid ethyl ester per ml, and 20 μg of lecithin per ml. Lipids and bilirubin were added to reaction cell 1 min prior to addition of mitochondria. Similar behavior was observed when β-hydroxybutyrate was replaced by succinate (cf. Fig. 4).

<table>
<thead>
<tr>
<th>Additions</th>
<th>No ADP</th>
<th>ADP</th>
<th>ADP:O ratio</th>
<th>Respiratory control index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. β-Hydroxybutyrate</td>
<td>0.10</td>
<td>0.96</td>
<td>2.80</td>
<td>9.5</td>
</tr>
<tr>
<td>2. β-Hydroxybutyrate + bilirubin</td>
<td>0.60</td>
<td>0.60</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>3. β-Hydroxybutyrate + mitochondrial lipid</td>
<td>0.10</td>
<td>0.95</td>
<td>2.70</td>
<td>9.4</td>
</tr>
<tr>
<td>4. β-Hydroxybutyrate + mitochondrial lipid + bilirubin</td>
<td>0.11</td>
<td>0.86</td>
<td>2.60</td>
<td>7.8</td>
</tr>
<tr>
<td>5. β-Hydroxybutyrate + asolectin</td>
<td>0.10</td>
<td>0.90</td>
<td>2.80</td>
<td>9.0</td>
</tr>
<tr>
<td>6. β-Hydroxybutyrate + asolectin + bilirubin</td>
<td>0.12</td>
<td>0.76</td>
<td>2.78</td>
<td>6.4</td>
</tr>
<tr>
<td>7. β-Hydroxybutyrate + ascites cell lipid</td>
<td>0.14</td>
<td>0.75</td>
<td>2.50</td>
<td>5.3</td>
</tr>
<tr>
<td>8. β-Hydroxybutyrate + ascites cell lipid + bilirubin</td>
<td>0.15</td>
<td>0.72</td>
<td>2.35</td>
<td>4.0</td>
</tr>
<tr>
<td>9. β-Hydroxybutyrate + triolein</td>
<td>0.10</td>
<td>0.90</td>
<td>2.60</td>
<td>8.2</td>
</tr>
<tr>
<td>10. β-Hydroxybutyrate + triolein + bilirubin</td>
<td>0.13</td>
<td>0.74</td>
<td>2.48</td>
<td>4.0</td>
</tr>
<tr>
<td>11. β-Hydroxybutyrate + vegetable oil</td>
<td>0.10</td>
<td>0.90</td>
<td>2.50</td>
<td>5.3</td>
</tr>
<tr>
<td>12. β-Hydroxybutyrate + vegetable oil + bilirubin</td>
<td>0.11</td>
<td>0.73</td>
<td>2.50</td>
<td>6.6</td>
</tr>
<tr>
<td>13. β-Hydroxybutyrate + linoleic acid ester</td>
<td>0.10</td>
<td>0.76</td>
<td>2.55</td>
<td>7.8</td>
</tr>
<tr>
<td>14. β-Hydroxybutyrate + linoleic acid ester + bilirubin</td>
<td>0.11</td>
<td>0.66</td>
<td>2.40</td>
<td>4.4</td>
</tr>
<tr>
<td>15. β-Hydroxybutyrate + lecithin</td>
<td>0.18</td>
<td>0.90</td>
<td>2.20</td>
<td>4.5</td>
</tr>
<tr>
<td>16. β-Hydroxybutyrate + lecithin + bilirubin</td>
<td>0.21</td>
<td>0.78</td>
<td>2.00</td>
<td>3.6</td>
</tr>
</tbody>
</table>

uncoupling was prevented, although respiratory control remained still somewhat affected as shown in Table I and Fig. 4. About 2 to 4 μg of lipid per ml were sufficient to overcome practically completely the uncoupling effect of bilirubin, while complete recovery of respiratory control was not achieved until the mitochondrial lipid concentration reached 10 μg per ml. Even at this level, asolectin was only partially effective. Respiratory control appears to be more sensitive than phosphorylation to the alteration

See the end of “Discussion.”
of mitochondrial membranes (see below). Protection of oxidative phosphorylation and related processes was also observed in the presence of many other lipids and lipoidal materials tested. 3

The rate of mitochondrial swelling caused by bilirubin was significantly diminished in the presence of external lipid; however, over-all swelling was not affected unless the lipid concentrations were high (cf. Table II). The differential degrees of protection by lipid against bilirubin toxicity, such as in respiratory control, phosphorylation, and swelling may indicate the differential degrees of essentiality of membrane integrity in these processes. The fact that external lipid was more effective in diminishing the rate rather than the over-all swelling would indicate a redistribution of bilirubin between the lipid of mitochondria and the lipid exogenously added.

A question can be raised whether or not these bilirubin effects show organization changes of the lipid structure of membranes. In view of the fact that serum albumin can reverse the binding of bilirubin to mitochondrial lipid, an answer to the above question might be found if bilirubin-treated mitochondria are made "free" of the bile pigment by washing with serum albumin and the washed mitochondria then examined to see whether or not they could regain normal function. Results from the experiments so designed are presented in Tables III and IV which show that bilirubin-treated mitochondria could be restored considerably to their normal functional state by washing with serum albumin.

The mitochondria of control experiments (Section A or B of Table III) gave an ADP:O ratio of 2.7 with a respiratory control index of about 9 for 3-hydroxybutyrate oxidation. The mitochondria (Section C, Table III) which were previously treated with bilirubin but freed of the pigment by washing with serum albumin gave an almost similar ADP:O ratio. The respiratory control indices, however, were low, about 50% of the control value. These results were in contrast to those presented in Section D of Table III, where mitochondria were previously treated with bilirubin but not washed with serum albumin. The respiratory rates were high being typical of an uncoupled state and there was no control whatsoever of respiration in the presence of ADP. We also conducted experiments with 3-hydroxybutyrate as a substrate and observed the same behavior as that with succinate.

From the above results it appears that the respiratory control was more sensitive than coupled phosphorylation (see also Reference 1). The following explanations might clarify whether the observation was apparent or genuine. In the mitochondria previously treated with bilirubin (Section C of Table III), the State 4 (basal respiration) rate was high, but the State 3 (phosphorylating respiration) rate was low compared to the corresponding rates in the untreated mitochondria of Section A or B. Obviously, the respiratory control index (a ratio of the rate in State 3 to that in State 4) would be low for the mitochondria of Section C. However, the extent of coupled phosphorylation (as expressed in terms of ADP:O ratio) was practically the same in all these mitochondria. But the rate of phosphorylation was not the same in these cases. The rate of active respiration was lower in the mitochondria of Section C, than in that of Section A or B, thus the actual rate of phosphorylation must be lower. Therefore, our conclusion is that the treatment of mitochondria with bilirubin followed by washing with serum albumin (as described in Table III) results in a partial loss of respiratory...
TABLE III

Restoration of functional state of bilirubin-treated mitochondria by serum albumin with oxidative phosphorylation as parameter

Basal medium was the same as in Table I, except for rat liver mitochondria, 1.2 mg (in terms of protein) per ml. Other additions as indicated were 10 mM succinate, 200 μM ADP, 22 μM bilirubin, and 6 mg of bovine serum albumin per ml. The prior treatment of mitochondria was made in 10 ml of a medium containing 0.28 M sucrose, 5 mM Tris-chloride, and 24 mg of protein, but in the absence of energy supply, in the following ways: A, untreated control; B, untreated but washed three times with 1% bovine serum albumin; C, treated with 44 μM bilirubin for 10 min and then washed three times with 1% bovine serum albumin; D, same as C, except washed only once with the medium.

<table>
<thead>
<tr>
<th>Type of mitochondria and additions</th>
<th>O₂ uptake</th>
<th>ADP:O ratio</th>
<th>Respiratory control index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No ADP</td>
<td>+ADP</td>
<td></td>
</tr>
<tr>
<td>A. Untreated control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Succinate</td>
<td>0.30</td>
<td>2.20</td>
<td>1.70</td>
</tr>
<tr>
<td>2. Succinate + bilirubin</td>
<td>1.76</td>
<td>1.76</td>
<td>0.00</td>
</tr>
<tr>
<td>3. Succinate + bovine serum albumin</td>
<td>0.28</td>
<td>1.90</td>
<td>1.70</td>
</tr>
<tr>
<td>4. Succinate + bovine serum albumin + bilirubin</td>
<td>0.30</td>
<td>1.82</td>
<td>1.69</td>
</tr>
<tr>
<td>B. Untreated but washed with bovine serum albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Succinate</td>
<td>0.30</td>
<td>2.12</td>
<td>1.70</td>
</tr>
<tr>
<td>2. Succinate + bilirubin</td>
<td>1.64</td>
<td>1.64</td>
<td>0.00</td>
</tr>
<tr>
<td>3. Succinate + bovine serum albumin</td>
<td>0.30</td>
<td>2.10</td>
<td>1.68</td>
</tr>
<tr>
<td>4. Succinate + bovine serum albumin + bilirubin</td>
<td>0.35</td>
<td>1.94</td>
<td>1.61</td>
</tr>
<tr>
<td>C. Treated and washed with bovine serum albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Succinate</td>
<td>0.40</td>
<td>1.62</td>
<td>1.64</td>
</tr>
<tr>
<td>2. Succinate + bilirubin</td>
<td>1.45</td>
<td>1.45</td>
<td>0.00</td>
</tr>
<tr>
<td>3. Succinate + bovine serum albumin</td>
<td>0.42</td>
<td>1.81</td>
<td>1.64</td>
</tr>
<tr>
<td>4. Succinate + bovine serum albumin + bilirubin</td>
<td>0.48</td>
<td>1.48</td>
<td>1.61</td>
</tr>
<tr>
<td>D. Treated but not washed with bovine serum albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Succinate</td>
<td>1.52</td>
<td>1.52</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Rate of swelling refers to an absorbance change for the 1st min and over-all swelling to a change in 6 min.

A. Untreated control
| Succinate | 0.01 |
| Succinate + bilirubin | 0.38 |

B. Untreated but washed with bovine serum albumin
| Succinate | 0.02 |
| Succinate + bilirubin | 0.35 |

C. Treated and washed with bovine serum albumin
| Succinate | 0.02 |
| Succinate + bilirubin | 0.34 |

D. Treated but not washed with bovine serum albumin
| Succinate* | 0.35 |

* No further addition of bilirubin was necessary.

The results presented here can afford to explain the adverse action of bilirubin on all four mitochondrial processes, namely, respiration, respiratory control, phosphorylation, and physical state. The binding of mitochondrial lipid by bilirubin alters the membrane resulting in a loss of its selective permeability to ions and metabolites. Thus, low concentrations of bilirubin stimulate respiration, giving rise to an uncoupled state of mitochondria. The uncoupling action of bilirubin on mitochondria and cells has been observed (1, 7-11) and in fact, the phenomenon has been referred to as an ion transport-dependent uncoupling (cf. Reference 1). When bilirubin concentrations are high enough, the lipid essential to respiratory enzymes is also bound by the bile pigment; consequently inhibition of respiration results. In fact, the inhibition of membrane-bound respiratory enzymes, e.g. NADH oxidase, succinate oxidase, and cytochrome c reductase, observed by other workers (7, 13) and also confirmed in our laboratory can be explained on the basis of this lipophilic behavior of bilirubin. The maintenance of the structural integrity of mitochondria and the manifestation of respiratory control depend upon the mitochondrial membrane. Since bilirubin binds to lipid, the membrane characteristics are altered and consequently, the abolishment of respiratory control and swelling of mitochondria result.

The reversal of binding between bilirubin and lipid by serum albumin is due to the high association constant of albumin for the bile pigment; the constant at pH 7.4 obtained by optical rotatory dispersion titration is at least 1 x 10⁷ M⁻¹ (31). Odell (14) has shown that bilirubin is concentrated within the micro-environment of mitochondria when they are suspended in a

control and somewhat of a diminution of the rate but not the extent of energy coupling.

Again, when swelling of mitochondria was used as a functional parameter (cf. Table IV), mitochondria of Section C behaved similarly to those of Section A or B, i.e., in the presence of an ion and energy source the addition of bilirubin was required for swelling to occur. This result was, again, in contrast to that of Section D, where mitochondria underwent swelling without further addition of bilirubin. These results indicate that the binding of bilirubin as well as its presence is required to manifest the adverse effects of this bile pigment on the aforementioned mitochondrial processes. Although bilirubin binding impairs membrane functions, the removal of the bile pigment seems to result in a considerable recovery of the normal structural as well as functional integrity of the mitochondrial membranes if it is done before the occurrence of irreversible destruction of the physical organization.

DISCUSSION
medium containing bilirubin, and that this association of bilirubin with mitochondria is prevented if the medium contained equimolar concentrations of bilirubin and albumin. In the treatment of neonatal jaundice, albumin in conjunction with exchange transfusion is sometimes used on the basis of this high affinity of serum albumin for bilirubin (e.g. References 19 and 20).

The binding of bilirubin to mammalian erythrocytes has been reported to cause a disruption of the ion gradient across the cell membrane with a loss of K⁺ and inorganic phosphate and an inhibition of glucose metabolism (32) (see however Reference 7). These effects have been explained as a result of bilirubin action on the surface of the cells. Red blood cells and tissues, especially cerebral tissue, can accumulate bilirubin (20, 33). Blanc and Johnson (34) assume that bilirubin is capable of entering into and interfering with the metabolism of ganglion cells. This view has been challenged by Haymaker et al. (35) who have claimed that only necrotic cells can take up bilirubin and that intracellular bilirubin is not the cause but a mere indication of cell necrosis. Cowger, Igo, and Labbe (7) have demonstrated that the chemical structure and the lipid solubility of bilirubin are responsible for its toxic effect, while the other bile pigments having a more polar character are less toxic. Similar possibilities have also been considered by other workers (36, 37) who have argued that nonpolar lipid-soluble molecules readily cross biological membranes including the blood-brain barrier, and that due to the lipid solubility, unbound bilirubin rapidly moves across such membranes as gut mucosa and placenta. Kahan, Timar, and Foldi (38), have reported that bilirubin specifically binds with cerebral gangliosides by means of which cerebral tissue can concentrate and retain the bile pigment, thus providing it the possibility to exert its toxic effects. However, our observation is that bilirubin can bind with many lipids or lipoidal material, although the degree of association might differ for various lipids. Consequently the micelles formed from different lipids may not be physically the same. Bilirubin has not been found to bind with mitochondrial protein.

In conclusion, we are inclined to think that the toxicity of bilirubin lies primarily in its lipophilicity. The mitochondrial as well as the cellular processes are basically the functions of organized membrane systems. Any alteration in the membrane organization is likely to impair the functions that are dependent on it. Indeed, the adverse effects on intact cells may be explained by the same general principle as applied for mitochondria in vitro.

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