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The Mechanism of Bilirubin Toxicity Studied with Purified Respiratory Enzyme and Tissue Culture Systems*

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ABSTRACT: The mechanism of bilirubin toxicity was studied in purified respiratory enzyme and whole cell systems. Like Amytal, a classical electron-transport inhibitor, bilirubin inhibited reduced nicotinamide-adenine dinucleotide oxidase but had little effect on succinate oxidase. Experiments with electron-transport fragments indicated that the locus of action of bilirubin was similar to Amytal. However, unlike Amytal, bilirubin had a powerful uncoupling effect on oxidative phosphorylation in whole mammalian cells in tissue culture.

Simultaneous measurements of oxygen uptake and glycolysis in intact cells further indicated that uncoupling was the primary toxic action of bilirubin on oxidative metabolism. Bilirubin also inhibited amino acid incorporation into protein, another property that distinguishes the mode of action of bilirubin from the barbiturates. Other bile pigments either had no effect or were effective only at greatly increased concentrations over that of bilirubin on electron-transport systems from purified enzymes or on whole cells in tissue culture.

The mechanism of bilirubin toxicity is poorly understood in spite of the interest this complex problem has received. The reward for its solution is twofold. Certainly there is academic interest in understanding the mode of action of any such potent biological inhibitor. However, there is also a very practical application in clinical medicine, that is, to help understand and perhaps prevent the brain damage that results in the newborn from the excessive accumulation of unconjugated bilirubin. The newborn infant lacks a significant level of activity of the enzyme, glucuronyl transferase, essential in the detoxication of bilirubin. This factor together with other circumstances unique to the newborn causes him to be especially vulnerable to this metabolic poison.

Previous workers have linked bilirubin toxicity to an effect on terminal oxidation. Day (1954) reported that bilirubin depressed the respiration of chopped rat brain. Cytochrome *c* reversed this inhibition. The same author, utilizing several different fresh tissue systems, extended these observations to include a series of tetrapyrrole pigments (Day, 1956). Bilirubin inhibited the

* From the Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington. *Received July 22, 1965; revised August 31, 1965.* This investigation was supported in part by a training grant (5-TI-GM-83) and a research grant (AM-02164) from the National Institutes of Health, U. S. Public Health Serivce, by an American Heart Association grant (64 G27), and by the Boeing Employees Good Neighbor Fund. Presented at the 48th Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 1964 (Cowger *et. al.*, 1964).

† This work was carried out during the tenure of an American Heart Association Advanced Research Fellowship which received partial support from the Washington State Heart Association.

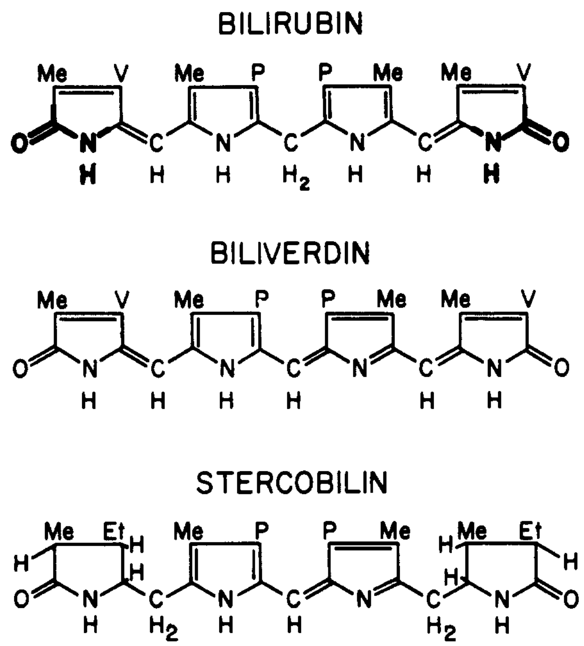

FIGURE 1: Molecular structures of bile pigments. The bold lettering in bilirubin corresponds to the molecular grouping presumed to function in the inhibition of $NADH_2$ oxidase.

respiration of rat brain, mouse diaphragm, and baker's yeast and the motility of protozoa. Hematin affected all systems except the protozoa. Mesobilirubin inhibited oxygen uptake by mouse diaphragm, but biliverdin had no inhibitory effects. Other investigators (Waters and Bowen, 1955; Bowen and Waters, 1957) compared the inhibitory effect of bilirubin in newborn and adult rat brain homogenates and noted that a concentration of 40 mg % showed a threefold greater inhibition of respiration in newborn than in adult brain. This inhibition could be reversed by nicotinamide–adenine dinucleotide (NAD).[1] Continuing their study of the toxic mechanism, but utilizing purified respiratory enzymes, Bowen and Waters (1958) found that bilirubin inhibited cytochrome *c* reductase and succinate oxidase but not reduced nicotinamide–adenine dinucleotide ($NADH_2$) oxidase. Zetterström and Ernster (1956) reported that bilirubin in a concentration of 3×10^{-4} M almost completely inhibited phosphorylation and partially depressed respiration in rat liver mitochondria when glutamate and α-ketoglutarate were used as substrates. The effect on respiration was less pronounced or absent with succinate, pyruvate, or β-hydroxybutyrate as substrates. The combined action of cytochrome *c* and NAD reversed the inhibition of respiration but did not restore oxidative phosphorylation. Biliverdin was not inhibitory in this system. Ernster (1961) presented additional evidence that bilirubin behaved as a detergent type of uncoupler of oxidative phosphorylation in rat liver mitochondria. Quastel and Bickis (1959) also noted an uncoupling effect in Ehrlich ascites tumor cells.

Observation of the structure of bilirubin suggested to us that this substance might be an inhibitor of $NADH_2$ oxidase in a manner analogous to the action of Amytal, a classical inhibitor of terminal electron transport. Amytal, in low concentrations, specifically inhibits the $NADH_2$ oxidase system in the area of the flavin and before the crossover point of ubiquinone, but at somewhat higher concentration it does affect the succinate oxidase system and oxidative phosphorylation (Chance and Hollunger, 1963). Previous studies from this laboratory dealing with structure–function relationships in a group of barbiturates and related compounds (Cowger *et al.*, 1962) indicated that the effective molecular structure for Amytallike inhibition consisted of a nonspecific hydrocarbon group attached to an amide, carbamide, or barbiturate, these compounds having in common the –CONH– group coupled to lipophilic groups. Further investigation of these compounds in tissue culture systems (Cowger *et al.*, 1963) showed that the same structural groupings inhibited oxidation in the intact cell. Figure 1 illustrates the resemblance of bilirubin to these drugs inasmuch as each terminal pyrrole group contains the necessary structure for $NADH_2$ oxidase inhibition. The second requirement for inhibition, that of lipid solubility, is also fulfilled by bilirubin. It therefore seemed appropriate in light of the metabolic effects previously elucidated for the Amytallike inhibitors to study further the effects of bile pigments on purified respiratory enzymes and mammalian cells.

Experimental Procedures

$NADH_2$ oxidase was prepared from beef heart mitochondria and assayed as described by Mackler and Green (1956). An electron-transport particle (ETP) that can catalyze the oxidation of succinate as well as $NADH_2$ by oxygen was prepared by a modification of the method of Crane *et al.* (1956). Its activity with succinate as substrate was measured at room temperature with an oxygen polarograph as described by Chance and Williams (1955). $NADH_2$–cytochrome *c* reductase and cytochrome oxidase were prepared and assayed using the method of Mackler and Penn (1957). The preparation and determination of $NADH_2$ dehydrogenase activity was as described by Mackler (1961).

Strain L-929 cells (Microbiological Associates, Albany, Calif.) were adapted to medium MB752/1, a protein-free medium containing 40 simple components (Waymouth, 1959). The cells were initially grown on a glass surface, then transferred to 1-l. spinner flasks and grown in suspension culture for 72–140 hr, depending upon the experimental purpose. Cell growth was based upon serial cell counts using a standard hemocytometer. Cells were stained with erythrocin B for viability counts. Metabolic experiments were conducted by harvesting the cells, washing once with 0.85% saline,

[1] Abbreviations used in this work: $NADH_2$, NAD, reduced and oxidized nicotinamide–adenine dinucleotide, respectively; ETP, electron-transport particle; ATP, adenosine triphosphate.

resuspending in a modified MB752/1 medium (glucose reduced by 50% and indicator omitted), and incubating for periods up to 4 hr. Total protein was estimated by a modified method of Lowry *et al.* (1951) and ATP by the luciferase method (Strehler and McElroy, 1957). Lactic acid determinations were made according to the method of Barker (1957), pyruvic acid by Natelson's method (1957), and glucose by the glucostat method (Worthington Biochemical Corp., Freehold, N. J.). Oxygen uptake of cells was carried out by incubating cell suspensions with varying concentrations of bilirubin and assaying on the oxygen polarograph as described above. Leucine-1-^{14}C uptake was determined by adding the isotope in the last 30 min of a 2-hr incubation of duplicate 30-ml cell suspensions. The cell suspension was then centrifuged, washed, and resuspended to 10 ml in saline. The saline suspension was sampled for determinations of cell count and protein content. Radioactivity of whole cell suspensions was determined by plating onto 2-in. planchets and counting with a low background detector. To the remaining cell suspension, 5 ml of 5% trichloroacetic acid was added. This was centrifuged and the precipitate was washed twice with 95% ethanol and once with ether. The residue was dissolved in 1 ml of concentrated HCl and the solution was plated, dried, and counted.

Bilirubin and biliverdin were commercial products (Nutritional Biochemical Corp., Cleveland, Ohio). Stercobilin was extracted from stool and recrystallized two times each from a methanol–ethyl acetate solvent and from a chloroform–ethyl acetate solvent. A 1×10^{-3} M solution of bilirubin was prepared immediately before use by dissolving 5.9 mg of bilirubin in 3.6 ml of 0.1 M Na_2CO_3 and diluting to 10 ml with 0.85% saline. Solutions of stercobilin were handled in a similar manner. However, biliverdin required 3 ml of 0.1 N NaOH for solubilization.

TABLE I: Effects of Bilirubin on the Activity of $NADH_2$ Oxidase and Succinate Oxidase.

Bilirubin Concn (M)	$NADH_2$ Oxidase Activity[a]	Succinate Oxidase Activity[a]
0	2.85	0.80
5.0×10^{-6}	2.20	
1.0×10^{-5}	1.85	
1.5×10^{-5}	1.28	0.90
2.0×10^{-5}	1.00	0.60
2.5×10^{-4}	0	0.40
1.5×10^{-5} + cytochrome *c*	1.30	
1.5×10^{-5} + albumin	2.90	

[a] Activities are expressed in μmoles of substrate oxidized/mg of protein per minute.

Results

The Effects of Bilirubin on Respiratory Enzymes. Fifty per cent inhibition of $NADH_2$ oxidase activity occurred at a bilirubin concentration of 1.0–1.5×10^{-5} M (Table I). Fifty per cent inhibition of succinate oxidase was not reached until a concentration of 2.5×10^{-4} M. The addition of cytochrome *c* did not restore activity of $NADH_2$ oxidase. The presence of albumin prevented completely the inhibitory effects of bilirubin.

The site of action of bilirubin inhibition was further localized using smaller fragments of the electron-transport chain (Table II). Bilirubin had no effect on cytochrome *c* oxidase. $NADH_2$–cytochrome *c* reductase activity was inhibited 40% at a bilirubin concentration of 1×10^{-5} M, 80% at 5×10^{-5} M, and 100% at 10×10^{-5} M. The activity of $NADH_2$ dehydrogenase, a soluble enzyme derived from the particulate system $NADH_2$ oxidase (Mackler, 1961), with indophenol as an electron acceptor, was progressively inhibited by greater

TABLE II: Effects of Bilirubin on Fragments of the Electron-Transport system.

Bilirubin Concn (M)	Cytochrome *c* Oxidase Activity[a]	$NADH_2$–Cytochrome *c* Reductase Activity[a]	$NADH_2$ Dehydrogenase Activity[a]	
			To Indophenol	To Cytochrome *c*
0	7.2	1.0	1.4	0.6
1.0×10^{-5}	7.4	0.6	1.2	0.7
5.0×10^{-5}	7.8	0.2	1.0	1.0
10.0×10^{-5}	7.2	0	0.6	1.0

[a] Activities are expressed in μmoles of substrate oxidized/mg of protein per minute.

TABLE III: Effect of Bilirubin on the "Opening Phenomenon."

Addendum	$NADH_2$ Oxidase Activity[a]	Cytochrome *c* Oxidase Activity[a]
None	2.5	0.5
Bilirubin (1.5×10^{-5} M)	1.0	0.5
Deoxycholate (0.1 mg/mg of protein)	1.0	10.0

[a] Activities are expressed in μmoles of substrate oxidized/mg of protein per minute.

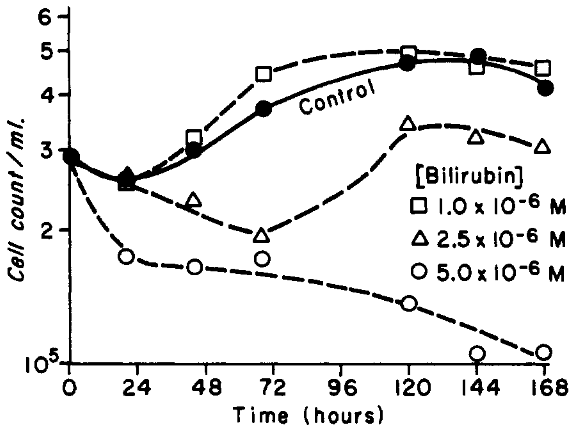

FIGURE 2: Effect of bilirubin on the growth of L-929 cells.

concentrations of bilirubin, while the same enzyme with cytochrome *c* as acceptor increased by 70% in activity with bilirubin present.

The possibility that bilirubin had a detergentlike action on $NADH_2$ oxidase was examined through its effect upon the "opening phenomenon" characteristic of detergents (Mackler and Green, 1956) (Table III). This phenomenon could not be demonstrated for bilirubin even though it was produced by deoxycholate in the enzyme preparation.

The effects of two other bile pigments on $NADH_2$ oxidase were also evaluated (Table IV). Biliverdin concentration required a 16-fold increase over the bilirubin concentration to cause 50% inhibition. Stercobilin was noninhibitory at 47 times the 50% inhibition level of bilirubin.

TABLE IV: Comparative Inhibition of $NADH_2$ Oxidase by Bile Pigments.

Bile Pigment	Concentration (M)	Inhibition (%)
Bilirubin	1.5×10^{-5}	50
Biliverdin	25.0×10^{-5}	50
Stercobilin	70.0×10^{-5}	0

Effects of Bilirubin on the Growth of Intact Mammalian Cells in Tissue Culture. Bilirubin was evaluated using intact, mammalian cells grown in tissue culture in a manner similar to that employed previously for studies of barbiturate action (Cowger *et al.*, 1963). Results of early experiments revealed that the usual culture media containing serum could not be used because of the extremely firm binding of bilirubin by serum albumin. Albumin-bound bilirubin exerted no apparent harmful effects on L-929 cells. Accordingly, this strain was adapted to protein-free media. This system in which very minute amounts of bilirubin are effective thus represents a physiological model in which the albumin binding has just been exceeded and free, unconjugated bilirubin becomes available to react with tissue cells.

Figure 2 represents the effect of bilirubin on growth, that is, increase in number of cells in suspension culture. At 144 hr bilirubin in a concentration of 1.0×10^{-6} M had no effect on growth. In a concentration of 2.5×10^{-6} M the cell number decreased by 34% and in a concentration of 5×10^{-6} M by 78% in the same growth period.

Effects of Bilirubin on Oxidative Metabolism. The effects of bilirubin on glucose utilization and lactate excretion by L-929 cells is depicted in Figure 3. The relationship between the two sets of curves is readily apparent. Since cell survival could greatly influence the degree of metabolic activity, this factor was observed. During the first hour of incubation cell viability remained the same in the control and the drug-treated cell suspensions. However, after this time viability began to decrease in the bilirubin-treated suspensions, depending partly upon the pH of the media as well as on the bilirubin concentration. In this experiment after 3-hr incubation the control culture remained 82% viable (the starting level). However, at a bilirubin level of 1.0×10^{-5} M viability had decreased to 25%, at 2.5×10^{-5} M to 10%, and at 5.0×10^{-5} M to 1%. Were these data then expressed in terms of viable cell number, the magnitude of the changes as well as the spread of values between individual bilirubin concentrations would be most remarkably altered, and in a way that would magnify those differences shown. Simultaneous determinations of lactate and pyruvate

TABLE V: Effects of Bilirubin on Glycolysis.[a]

Determination	Bilirubin Concentration (M) 0	1.0×10^{-5}	2.5×10^{-5}	5.0×10^{-5}
Glucose utilization				
mmoles/liter	0.149	0.205	0.211	0.355
% increase		38	42	138
Pyruvate excretion				
mmoles/liter	0.032	0.026	0.028	0.034
Lactate excretion				
mmoles/liter	0.168	0.321	0.394	0.492
% increase		91	135	193
mmoles of "excess lactate"/liter		0.184	0.247	0.334
% of total lactate		57	63	68

[a] Data taken from experiment in Figure 3 following 60-min incubation. "Excess lactate" refers to that derived from anaerobic metabolism (Huckabee, 1958).

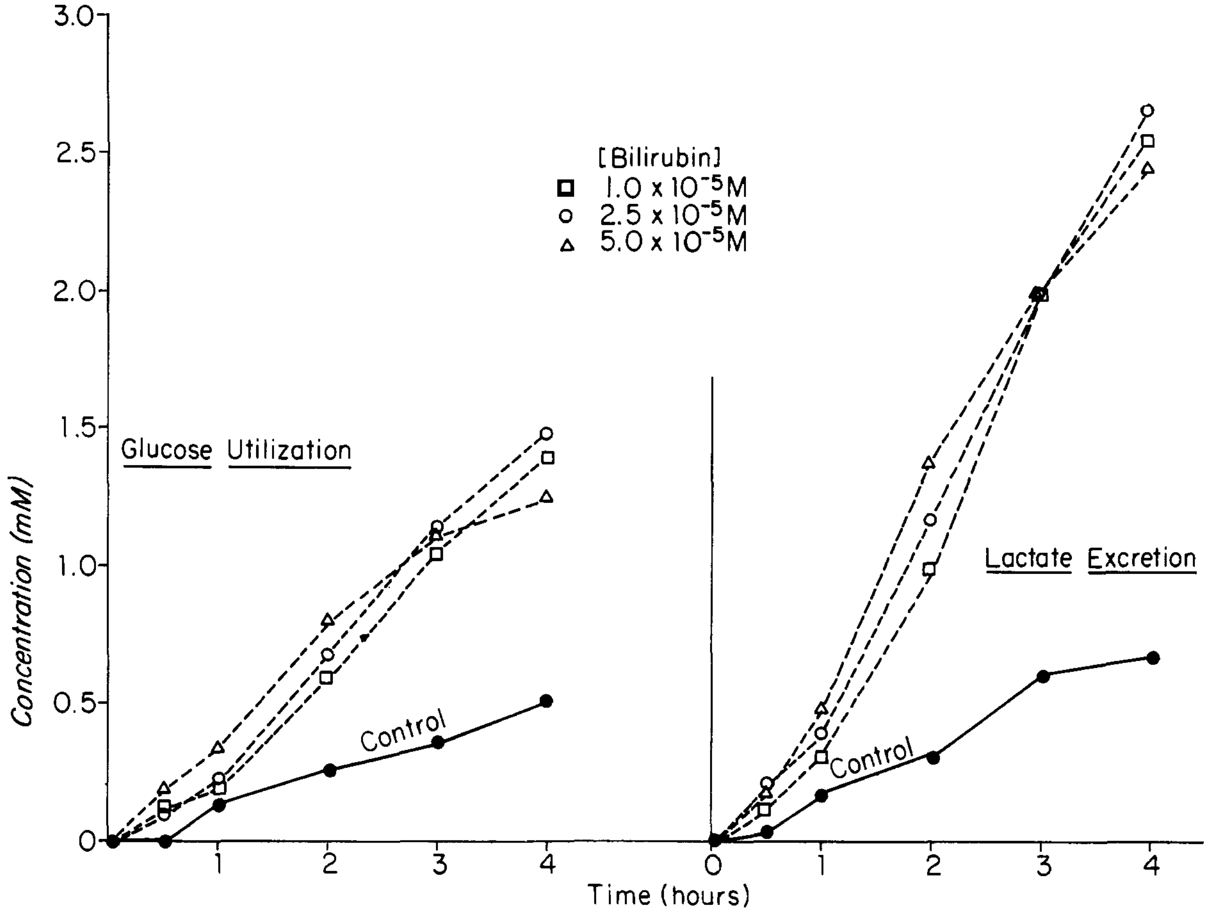

FIGURE 3: Effect of bilirubin on lactate excretion and glucose utilization by L-929 cells.

TABLE VI: Oxygen Uptake of Tissue Culture Cells as Influenced by Varying Concentrations of Bilirubin.

Bilirubin Concn (M)	Initial Rate of Oxygen Uptake (mμmoles/mg protein per min)	Initial Rate of Oxygen Uptake (% increase)	Total Lactate Production (% increase)
0	5.6		
5.0×10^{-6}	8.0	42	6
7.5×10^{-6}	8.2	45	15
1.0×10^{-5}	9.2	63	17
2.5×10^{-5}	8.6	54	43

excretion allow one to calculate the per cent of "excess lactate," or that amount of lactate produced which can be attributed to anaerobic conditions (Huckabee, 1958). These data obtained at the end of incubation for 1 hr are shown in Table V. One hour was chosen in order to make a more meaningful comparison at a time when the cell viability was similar in all flasks. The "excess lactate" makes up 57% of the total lactate excreted at a bilirubin concentration of 1.0×10^{-5} M, 63% at a concentration of 2.5×10^{-5} M, and 68% at a bilirubin level of 5.0×10^{-5} M. Biliverdin and stercobilin in a concentration of 2.5×10^{-5} M did not increase lactate production significantly.

The effects of bilirubin and biliverdin on cellular ATP can be seen in Figure 4. At the end of 2 hr a bilirubin concentration of 2.5×10^{-5} M caused cellular ATP to decline by 76%. Biliverdin in a similar concentration did not affect cellular ATP levels. These changes occurred during a time interval when cell viability had changed very little in any of the flasks.

Oxygen uptake of cultured cells was determined by preincubating cells with various concentrations of bilirubin for 1 hr prior to observing the behavior of these cells with an oxygen polarograph (Table VI). In concentrations as low as 5.0×10^{-6} M initial oxygen uptake increased by 42%. This appeared to reach a maximum of 63% at 10^{-5} M. In a subsequent experiment oxygen uptake was undetectable with 5.0×10^{-5} M bilirubin. Of particular significance is the inverse relationship between oxygen uptake and lactate production, that is, when the former begins to diminish at 2.5×10^{-5} M bilirubin the latter markedly rises.

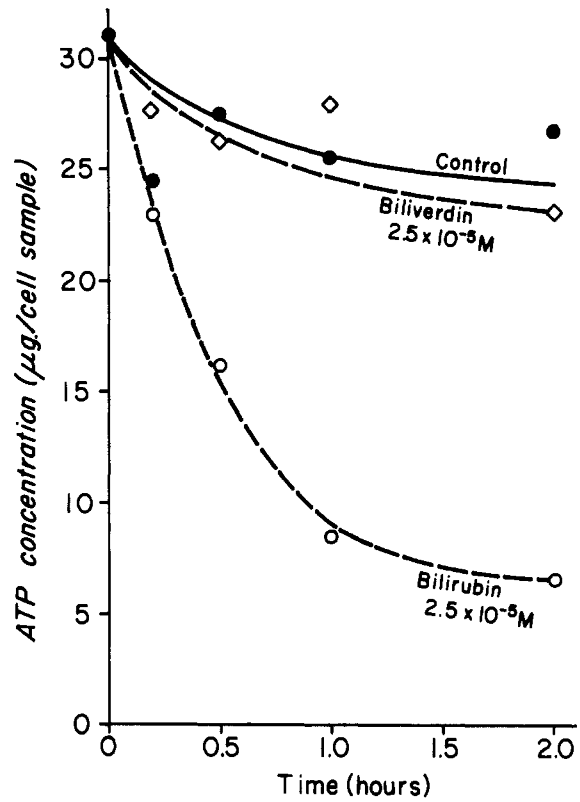

FIGURE 4: Effects of bilirubin and biliverdin on cellular ATP.

TABLE VII: Leucine-1-^{14}C Uptake and Incorporation into Cellular Protein.

Addendum	10^{-3} cpm/μg protein	% Change	10^{-6} cpm/Cell	% Change
Control	106		114	
Bilirubin 5.0 × 10^{-6} M	78	↓ 26	95	−17
Bilirubin 1.0 × 10^{-5} M	51	↓ 52	71	−38
Bilirubin 2.5 × 10^{-5} M	32	↓ 66	55	−51
Amytal 1.0 × 10^{-3} M	93	↓ 12	113	0

Effects of Bilirubin on Protein Synthesis. Table VII represents the effect of bilirubin and Amytal on the cellular uptake and incorporation into protein of leucine-1-^{14}C. Bilirubin in a concentration of 5.0 × 10^{-6} M decreased uptake and conversion of this amino acid into trichloroacetic acid precipitable cellular protein by 26%, at a concentration of 10^{-5} M by 52%, and at 2.5 × 10^{-5} M by 66%. Amytal in this same period of time and at nearly 50 times the concentration had no significant effect.

Discussion

The experiments described not only confirm that bilirubin is an extremely powerful metabolic poison but they also point out the importance and value of using multiple kinds of experimental systems in determining the mode of action of such a biological inhibitor. Thus, bilirubin had a specific effect on a purified enzyme system which apparently was of secondary importance in the intact cell. Albumin prevented the inhibitory effect of bilirubin in both systems, *i.e.*, in the intact cell and in a purified respiratory enzyme. This is not surprising and is in fact predictable from the information known concerning the importance of albumin-binding in bilirubin metabolism and the significance of bound bilirubin in the prevention of toxicity (Odell, 1959).

Bilirubin was a more effective inhibitor of purified $NADH_2$ oxidase than Amytal, a classical inhibitor of this enzyme system. Like Amytal, bilirubin had little effect on the succinate oxidase system until the concentration had been raised considerably. These results contradict those of Bowen and Waters (1958). However, our results based upon fragments of the electron-transport system are consistent with the findings of inhibition in the more complete particle, $NADH_2$ oxidase. There was no inhibitory effect on cytochrome *c* oxidase, demonstrating that the latter part of the respiratory chain was not involved. $NADH_2$–cytochrome *c* reductase is progressively inhibited by increasing concentrations of bilirubin, localizing the involved area in the NAD portion of the chain before cytochrome *c*. Bowen and Waters (1958) also reported the inhibition of cytochrome *c* reductase, which is to be expected with an Amytallike inhibition of $NADH_2$ oxidase. Referring to $NADH_2$ dehydrogenase, progressive inhibition occurred when indophenol was used as an acceptor but inhibition did not occur when cytochrome *c* was the acceptor, a finding consistent with the effect of Amytal on this particular enzyme (Mackler, 1961). Amytal inhibited the activity when indophenol and flavin–adenine dinucleotide were acceptors but did not affect the reactions with oxygen and cytochrome *c* as acceptors. This again argues for a strong similarity between bilirubin and Amytal inhibition of terminal oxidation. It should be pointed out that the activity of purified $NADH_2$ dehydrogenase with cytochrome *c* as acceptor differs from that of the particulate systems in that the former is not antimycin sensitive.

The addition of cytochrome *c* to $NADH_2$ oxidase did not prevent inhibition by bilirubin. Using chopped rat brain, Day (1954) reported a reversal of inhibition by cytochrome *c*, although this compound did not afford a protective effect on newborn rats given bilirubin (Day, 1956). Zetterström and Ernster (1956) utilizing rat liver mitochondria noted a reversal of bilirubin inhibition by a combination of NAD and cytochrome *c* on respiration, but no effect in restoring phosphorylation.

In tissue culture cells, bilirubin caused the rate of glycolysis to increase in intact cells as demonstrated by the increase in lactate production and a parallel increase in glucose utilization. Simultaneously cellular ATP was shown to decrease. These results were similar to those of previous studies in tissue culture with Amytal and related compounds. However, oxygen uptake of cell suspensions was depressed by the addition of Amytal except in the presence of succinate. Oxygen uptake studies with bilirubin on the contrary demonstrated an initial increase, but at higher concentrations a depression of respiration occurred. As oxygen uptake began to diminish lactate production significantly increased. This all suggests that initially the predominant or at least the primary effect of bilirubin was to uncouple oxidative phosphorylation. As the concentration increased, Amytallike inhibition of $NADH_2$ oxidase became more significant and eventually blocked respiration in this portion of the chain altogether. A primary uncoupling effect is in agreement with Zetterström and Ernster's earlier work (1956).

Ernster (1961) postulated that bilirubin was behaving as a detergent type of uncoupler. The effect of bilirubin on the "opening phenomenon," a response of $NADH_2$ oxidase to detergents, was investigated (Mackler and Green, 1956). This phenomenon is characterized by a progressive loss of $NADH_2$ oxidase activity and a reciprocal increase in cytochrome *c* oxidase activity. The amount of bilirubin and deoxycholate chosen was sufficient to cause more than 50% inhibition of $NADH_2$ oxidase activity. Bilirubin did not open the enzyme, suggesting that it did not function as a detergent in this particular regard. Ernster's results were obtained in a different system using rat liver mitochondria. The mechanism of uncoupling deserves further investigation.

Bilirubin appeared to resemble the effect of Amytal in being a more effective inhibitor of $NADH_2$ oxidase than of succinate oxidase. However, it differed from Amytal by seeming to be a more effective uncoupler of oxidative phosphorylation, as noted by decreased ATP production and increased oxygen uptake in whole cells. This effect seemed to predominate over $NADH_2$ oxidase inhibition. It differed in yet another way. Barbiturates have been reported to increase the uptake of amino acids and incorporation into cellular protein of cell-free liver preparations. Mitochondria, microsomes, and cell sap all contributed to this effect (Gelboin and Sokoloff, 1961). By contrast, bilirubin markedly depressed the uptake and incorporation of leucine-1-^{14}C into cellular protein in intact cells. Amytal at many times the concentration of bilirubin during this same short term experiment did not change the uptake of leucine into the whole cell although lactate excretion had increased, suggesting that inhibition of electron transport had occurred. A longer term study of Amytal with whole cells might reveal an effect on amino acid uptake, and this point perhaps deserves further study. The effect of bilirubin on the uptake of amino acids was previously noted by Quastel and Bickis (1959), who reported a decrease in the uptake of ^{14}C-glycine by Ehrlich ascites cells treated with bilirubin.

The two other bile pigments studied, biliverdin and stercobilin, did not affect tissue culture cells in concentrations at which bilirubin had devastating effects. This difference in toxicity of the other bile pigments can be explained on theoretical grounds. Gray *et al.* (1961) carried out a series of spectrophotometric titrations which revealed the predominantly basic character of stercobilin, the urobilins, and the verdins, the basic strength decreasing in that order, and a predominantly acidic character in the rubins. According to Gray, the keto form of these compounds is chemically a more correct way to portray them (Figure 1). The stronger basic characteristics of stercobilin are attributed to the saturated nature of the terminal rings. The pyrroline nitrogen, being undisturbed by effects from remote parts of the molecule, is available for external coordination. Hydrogen bonding can also occur between the two terminal oxygen atoms, thus removing the inhibitory structure. The verdins are much weaker bases because of the extensive resonating system. The nucleophilic nitrogen atoms are less likely to be externally coordinated, and hydrogen binding is less likely to occur between the two terminal oxygens. The difference in structure of these two compounds is reflected in the enzyme data. Stercobilin had no inhibitory effect, but biliverdin had some effect at increased concentrations. Bilirubin has no nucleophilic nitrogen, and this is reflected in the fact that it does not form hydrochlorides or metal complexes and it is further stabilized by the negative carbonyl groups. Bilirubin thus lacks the basic character of the other bile pigments. The bislactam form is more compatible with its chemical characteristics, and two inhibitory groups on the molecule are free to interact.

Tissue culture provides a system to study the effects of inhibitors on cellular growth both in terms of cell number and cell diameter. The Amytallike inhibitors have been observed to decrease cell number but increase cell diameter (Cowger *et al.*, 1963; Dale and Fisher, 1958). Bilirubin had a profound effect on growth, decreasing cell number in concentrations as low as 2.5 $\times 10^{-6}$ M (Figure 2). Interestingly, the initial cell count at this concentration decreased, and then after 72 hr rose again above the starting level. Perhaps the cells most affected by the drug died, clearing the medium of the nonprotein-bound bilirubin by attachment to cellular protein, and the remaining viable cells were present in a high enough concentration to initiate growth once again. Progressive cell death occurred at a bilirubin concentration of 5.0×10^{-6} M. Measurements of cell diameter of bilirubin-treated cells did not give any conclusive results. Kikuchi (1961) has made a more exhaustive study of some of the effects of bilirubin on growth of a number of cell lines. Strains of L-929 and HeLa cells adapted to protein-free media were affected by bilirubin, but serum-grown strains were not. It is interesting to note that ten times more bilirubin was required to affect significantly the growth of HeLa cells than of L-929 cells.

Tissue culture systems are also useful in obtaining some estimation of the effects of drugs on cell viability. In this regard it might be pointed out that bilirubin again has quite a different effect than those drugs that inhibit primarily $NADH_2$ oxidase. Bilirubin causes a rapid loss of viability as measured by cellular uptake of erythrocin B. This effect upon viability is partly dependent upon the pH of the incubation medium, viability decreasing markedly beginning within the first hour of incubation when the pH is less than 7.6. The decrease in viability does not occur with the Amytal-like drugs either in short-term experiments or in long-term growth of cells in suspension culture. The depression of growth apparent with these drugs probably results from lack of cell division. The cell death resulting from bilirubin thus may be attributable to the effect of uncoupling oxidative phosphorylation rather than inhibited respiration or perhaps to some as yet unknown toxic effect on metabolism. This point will be investigated further in tissue culture utilizing certain drugs that are primarily uncouplers and noting their effects on cell growth, viability, and cell metabolism.

Acknowledgment

The authors wish to thank Margaret Sewell for invaluable technical assistance throughout this investigation.

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