Studies *in vivo* of the effect of unconjugated bilirubin on hepatic phosphorylation and respiration¹

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EDITORIAL SYNOPSIS Unconjugated bilirubin is believed to be toxic to the brain in neonates. Previous work *in vitro* has shown an important effect on depression of cell respiration and phosphorylation. The present study *in vivo* fails to confirm these findings in the liver. The exact mechanism of kernicterus remains uncertain.

Studies *in vitro* of various tissues, including liver, have shown that unconjugated bilirubin at high concentrations uncouples phosphorylation from oxidation (Ernster, 1961). It has accordingly been postulated that the unconjugated bilirubin exerts its cytotoxic effect in kernicteric brain (Ernster, 1961), and possibly in intensely hyperpigmented liver (Brown, Grodsky, and Carbone, 1964; Rozdilsky, 1961) *in vivo* by interfering with the synthesis of adenosine triphosphate (ATP) which is essential for the proper metabolic function of these organs.

The present study provides an assessment of the effect of high levels of unconjugated bilirubin *in vivo* on hepatic ATP concentration and respiration. The results of this investigation, apparently the first one in living animals, are the basis of this report.

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURE Non-fasted 1- and 2-day-old guinea-pigs lightly anaesthetized with ethyl ether were infused via the inferior vena cava with 1 ml. of a solution of unconjugated bilirubin (Eastman Kodak, Rochester, New York), in physiological saline (2-5 mg./ml.), freshly prepared as described previously (Schenker and Schmid, 1964). Control litter mates received either the pigment-free carrier solution or were sham-injected. Sixty minutes later liver was obtained from both experimental and control animals for measurement of ATP concentration or oxygen consumption and plasma was obtained for determination of total and direct-reading bilirubin concentration. In additional studies similar pigment loads of biosynthesized unconjugated bilirubin-¹⁴C (Ostrow,

Hammaker, and Schmid, 1961) were infused into newborn guinea-pigs and the distribution of the isotope at the end of 60 minutes was determined in serum, liver, excreted bile, as well as in brain.

To assess the effect of prolonged unconjugated hyperbilirubinaemia on hepatic ATP concentration, the nucleotide was also assayed in livers of Gunn rats, a Wistar strain with hereditary unconjugated hyperbilirubinaemia due to a deficiency of glucuronyl transferase, and with onset of jaundice shortly after birth (Johnson, Sarmiento, Blanc, and Day, 1959). Three groups of these animals were studied: (1) Eight rats, aged 2 days to 9 months, with endogenous hyperbilirubinaemia of less than 10 mg./100 ml.; (2) three adults infused with unconjugated bilirubin in saline three and one half hours previously with resultant serum unconjugated bilirubin levels of over 20 mg./100 ml.; (3) two 16-day-old animals with kernicterus. Comparable control studies were performed in non-icteric litter mates of the Gunn rats and in normal Wistar rats of the same age, both uninjected and those infused with bilirubin-free saline. In all animals, except the kernicteric ones, total and direct-reacting plasma bilirubin was measured at the end of the experimental period. In the bilirubin-infused Gunn rats, a pre-injection, and in two instances serial, plasma bilirubin levels were determined on blood collected from the tip of the tail.

METHODS OF ANALYSIS Bilirubin concentration in administered solutions and plasma was measured by the diazo reaction (Malloy and Evelyn, 1937). Since only a terminal sample could be obtained from newborn guinea-pigs without disturbing their circulation, their plasma bilirubin concentration immediately after infusion of the pigment was estimated by dividing the quantity of bilirubin injected by the animal's plasma volume (Schenker and Schmid, 1964) plus the volume of the infusate. In three instances, the validity of the calculation, which falsely assumes equilibration and retention of all the bilirubin in the circulation, was checked by measuring plasma bilirubin concentration 10 minutes after infusing a similar pigment load (allowing equilibration) and

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comparing the results with the calculated value. The measured concentration was about 80% (range 71.9-91.5%) of the predicted value, indicating that the latter was not much above the actual plasma bilirubin level.

For determination of ^{14}C isotope in plasma and tissues, these were prepared for radioassay in a tricarb scintillation counter as described previously (Ostrow *et al.*, 1961). Internal standard was used to correct for quenching and the results converted to disintegrations per minute and expressed as a percentage of injected radioactivity. In a control study, the extraction procedure used (which does not permit separate measurement of unconjugated and conjugated bilirubin fractions) recovered 93% of radioactivity added to liver in the form of unconjugated bilirubin- ^{14}C saline solution.

The ATP was measured on the rapidly frozen liver by the luciferin-luciferase-luminescence method (Schenker and O'Donnell, 1965). All assays were carried out in duplicate and the results averaged. The technique and its validation and reproducibility under these experimental conditions have been described in a prior publication (Schenker and O'Donnell, 1965). The ATP concentration was expressed both as micromoles per gram liver, wet weight, and per gram liver protein, the latter having been determined for each specimen by the method of Lowry, Rosebrough, Farr, and Randall (1951). Earlier studies (Schenker and O'Donnell, 1965) have established that sex of the animals, the site of liver sampling, and the presence or duration (after five minutes) of ether anaesthesia have no effect on hepatic ATP levels, hence these factors were not separately assessed. Addition *in vitro* of a quantity of unconjugated bilirubin, calculated to be present in liver from isotope studies, to newborn guinea-pig liver, failed to affect the hepatic ATP concentration as compared with adjacent hepatic tissue assayed without added bile pigment.

For determination of oxygen consumption, a section of liver weighing about 1.2 g. was rapidly excised, weighed, and finely homogenized by hand with 5 volumes/weight of 0.04 M KH_2PO_4 buffer adjusted to pH 7.6 with 0.1 N NaOH. The above procedure was carried out in a pre-cooled glass homogenizer maintained throughout in crushed ice and the timing was set at exactly one and one-half minutes from tissue excision to the start of homogenization which was completed in another four and one-half minutes. At this time 1.4 ml. of the homogenate was pipetted rapidly into a prechilled Warburg flask to which was added another 0.4 ml. of phosphate buffer and 0.2 ml. of 10 mg./ml. glucose. In the centre well were placed 0.2 ml. of 10% KOH and a strip of folded filter paper to enhance the absorption of liberated carbon dioxide. The flasks, placed on ice, were gassed for five minutes with pure oxygen and after an equilibration of 10 minutes, oxygen consumption was measured by standard Warburg manometry at 10-minute intervals for one hour. The incubation was carried out at 37°C., at 50 strokes per minute. All studies were carried out in triplicate, the results averaged and expressed in microlitres (μl .) of oxygen consumed/mg. tissue (wet weight)/l hour. In most instances the protein concentration of the experimental and control homogenates was determined and the results also expressed on basis of liver protein.

Demineralized distilled water was used for preparation of all solutions and the washing of glassware.

ANCILLARY STUDIES To assess interlitter mate variability with respect to hepatic oxygen consumption, this was investigated in two litters of three and four 1-day-old guinea-pigs, uninjected but anaesthetized with ether for 60 minutes. Similarly, the effect of intravenous saline on hepatic respiration was studied in injected and uninjected newborn litter mate guinea-pigs.

In three instances, the effect of unconjugated bilirubin *in vitro* on hepatic oxygen consumption was determined. For this the incubation system described above was employed with the following modifications: all liver tissue was obtained from uninjected 1-day-old guinea-pigs; 30 mg. of commercial bilirubin was dissolved in 0.3 ml. of 0.1 N NaOH and 9.6 ml. of phosphate buffer and 0.2 ml. of the final solution (containing 600 μg . of bilirubin) was added to the Warburg flask immediately before incubation; bilirubin-free buffer was added to the control flasks containing liver homogenates. This incubation medium was closely patterned after one used previously by Waters and Bowen (1955) to show a depressing effect of unconjugated bilirubin *in vitro* on rat brain homogenates.

RESULTS

The effect of unconjugated hyperbilirubinaemia on hepatic ATP concentration of newborn guinea-pigs is presented in Table I. The plasma unconjugated bilirubin levels achieved during the experimental period were well within, and in some instances in excess of, the range of values reported for kernicteric infants (Hsia, Allen, Gellis, and Diamond, 1952) and those used *in vitro* to demonstrate a depressant effect of the pigment on tissue phosphorylation (Ernster, 1961). Despite this, hepatic ATP levels, both on the basis of tissue wet weight and its protein concentration, were similar in bilirubin-infused and control litter mates. In addition, the nucleotide concentration in the icteric group closely correspond to that obtained in another series of 30 1- and 2-day-old control guinea-pigs (Table I). In the bilirubin-infused animals there was no correlation between the height of the initial or terminal plasma bilirubin level and the ATP concentration in liver.

A comparable degree of hyperbilirubinaemia of one hour's duration likewise failed to affect the hepatic oxygen consumption of newborn guinea-pigs (Table II). Comparison of rates of oxygen consumption (at 10-minute intervals) also revealed similar values in the experimental and control groups. The inter-litter mate variation in hepatic oxygen consumption among normal sham-injected newborn guinea-pigs was $\pm 10\%$ and this was unaffected by the infusion of pigment-free saline. Addition of unconjugated bilirubin (final concentration of 30 mg./100 ml. medium) to liver homogenates in the

TABLE I

EFFECT OF UNCONJUGATED HYPERBILIRUBINAEMIA FOR 60 MINUTES ON HEPATIC ATP LEVELS OF NEWBORN GUINEA-PIGS

Litter No.	Animal Age (days)	Bilirubin Administered		Total Serum Bilirubin (mg./100 ml.) ¹		Hepatic ATP Level			
		mg.	$\mu\text{g./g.}$ Body Weight	Start ¹	End	Bilirubin-infused		Control ²	
						$\mu\text{M/g. Liver}$ (wet weight)	$\mu\text{M/g. Liver Protein}$	$\mu\text{M/g. Liver}$ (wet weight)	$\mu\text{M/g. Liver Protein}$
1	1	4.6	49.5	73.0	25.5	2.42	17.06	2.82	23.22
2	1	5.0	53.2	78.7	62.0	3.28	17.20	3.14	18.51
3	1	7.0	75.3	111.1	56.0	3.91	27.59	3.68	24.57
4	2	2.5	23.4	35.2	14.4	3.98	21.63	3.73	18.54
5	2	2.5	27.2	40.3	8.8	3.18	18.19	3.02	21.78
6	2	2.8	30.4	45.2	11.0	3.33	21.68	3.04 ⁴	19.66 ⁴
7	2	4.0	45.5	66.4	18.0	2.81	14.37	2.64 ⁴	14.35 ⁴
	Mean	4.06	43.50	64.27	27.95	3.27	19.67	3.15	20.12
	$\pm$ S.D.					± 0.52	± 4.03	± 0.38	± 3.11
Hepatic ATP level, mean $\pm$ S.D. of control guinea-pigs ⁵						18 one-day-old guinea-pigs			
						20.61			
						12 two-day-old guinea-pigs			
						3.26			
						19.73			
						± 3.46			

¹Bilirubin concentration at the start of the experiment is estimated from the quantity of pigment infused and the animals' plasma volume (4) plus the volume of infusate. Equilibration of the bilirubin in plasma and lack of diffusion into tissues are assumed (see Materials and Methods).²Only a trace or no direct-reacting bilirubin was detected.³Control animals, unless otherwise specified, were sham-operated litter mate guinea-pigs (see Materials and Methods). Their serum at sacrifice revealed only a trace or no detectable bilirubin.⁴Animals infused with 1 ml. of pigment-free physiological saline.⁵The data for these normal unoperated newborn guinea-pigs have been included in a prior publication (*Amer. J. Physiol.*, 208, 573, 1965). They have been here adjusted downward by 16.47% to correct for products of crystallization present in the previously used standard crystalline ATP.

TABLE II

EFFECT OF UNCONJUGATED HYPERBILIRUBINAEMIA ON HEPATIC OXYGEN CONSUMPTION OF NEWBORN GUINEA-PIGS

Litter No.	Animal Age (days)	Bilirubin Administered ¹		Total Serum Bilirubin (mg./100 ml.)		Hepatic Oxygen Consumption ($\mu\text{l./mg. liver/hr.}$) ⁴	
		mg.	$\mu\text{g./g. Body Weight}$	Start ¹	At One Hour ²	Bilirubin-infused	Control ³
1	1	4.1	47.7	69.5	23.0	1.11	0.88
2	2	2.4	32.4	46.0	28.0	1.26	1.01
3	2	3.6	33.6	50.7	10.0	1.01	1.14
4	2	4.8	38.4	59.0	25.0	0.76	0.61
5	2	2.5	39.1	53.8	14.0	0.61	0.37
6	2	4.0	40.8	60.7	20.0	0.73	0.95
7	2	2.6	42.6	58.0	24.2	0.85	1.14
8	2	5.4	74.0	104.7	57.0	0.49	0.51
	Mean	3.68	43.58	62.80	25.14	0.853	0.826
	$\pm$ S.D.					± 0.244	± 0.275

¹The unconjugated bilirubin was administered in 1 ml. of physiological saline.²See pertinent footnote to Table I.³Only a trace or no direct-reacting bilirubin was detected.⁴The hepatic oxygen consumption is expressed in terms of tissue wet weight. Protein determination on tissue homogenates and calculation of data on that basis yielded similar results.⁵Control animals were infused with 1 ml. of pigment-free physiological saline and at sacrifice the serum revealed only a trace or no detectable bilirubin.

same incubation system, however, resulted in a 30-50% decrease in oxygen consumption, as compared with litter mate liver assayed without added pigment.

Table III shows the distribution of ¹⁴C radioactivity in newborn guinea-pig plasma, liver, and bile after administration of unconjugated bilirubin-¹⁴C in amounts comparable to those employed above. The quantity of radioactivity retained in the circulation was obtained from the product of the isotope concentration in plasma and the plasma volume

(Schenker and Schmid, 1964). In two instances, plasma bilirubin was measured also by the diazo reaction and the quantity retained in plasma, as a percentage of injected pigment, correlated well with calculations based on radioassay. Only traces of direct-reading bilirubin were found in plasma of bilirubin-infused newborn guinea-pigs. The mean isotope at the end of 60 minutes in plasma and liver, disregarding the small contribution of trapped intra-hepatic plasma (Grodsky, Contopoulos, Fanska, and Carbone, 1963) and bile (Barber-Riley, 1963)

TABLE III

DISTRIBUTION OF ADMINISTERED UNCONJUGATED BILIRUBIN-¹⁴C IN NEWBORN GUINEA-PIGS

Animal Age (days)	Bilirubin- ¹⁴ C Administered		Percentage Administered Radioactivity ¹		
	mg.	μg./g. Body Weight	Retained in Plasma	Retained in Liver ²	Excreted in Bile
1 ^a	2.3	21.3	12.1	21.6	24.7
1 ^a	2.3	23.7	7.4	30.8	22.4
1	5.0	39.1	31.9	27.9	8.0
1	5.0	41.3	32.2	21.7	6.8
2	1.4	16.9	8.8	8.6	43.0
2 ^a	3.2	34.4	35.6	19.4	8.9
2 ^a	4.1	44.1	38.9	19.1	9.3
Mean	3.33	31.54	23.84	21.31	17.59

¹Radioassay carried out 60 minutes after administration of labelled bilirubin.²Estimate of total bilirubin. Liver extracted for radioassay with 1 N NaOH (see Materials and Methods). No correction was made for the plasma and bile trapped in liver.^aLitter mates.

which, extrapolating from rat data, constitute less than 1% of injected label, was 23.8 and 21.3% of injected radioactivity, respectively. Inasmuch as liver, assessed in nine animals, and plasma constitute, respectively, 4.3 and 5.7% of newborn guinea-pig weight, it appears that the isotope concentration in both compartments was similar on average.

A mean of 17.6% of infused labelled bilirubin, equal to 441.6 μg. of the pigment, was excreted in bile in one hour. In separate experiments with newborn guinea-pigs, provided with external biliary fistulae, it was established that virtually all of the freshly collected bilirubin in bile reacted directly with the diazo reagent. While biliary excretion of the bilirubin was determined only from isotope content of the gall bladder and gastrointestinal tract, radio-

assay of bile collected by external biliary drainage has confirmed the validity of the present technique (Schenker and Schmid, 1964). In addition, similar studies in adult guinea-pigs, with concurrent estimation of biliary bilirubin-¹⁴C, have shown that at least 75% of biliary ¹⁴C radioactivity is excreted in the form of the labelled conjugated bilirubin (Schenker, Dawber and Schmid, 1964). In this study, only traces of the isotope were found in the brain of newborn guinea-pigs.

As shown in Table IV, prolonged endogenous unconjugated hyperbilirubinaemia, albeit low grade, also had no depressant effect on Gunn rat hepatic ATP concentration. In addition, three bilirubin-infused adult Gunn rats with plasma bilirubin levels of 25 to 30 mg./100 ml. at the end of three and one-half hours (and higher in the intervening period) and two kernicteric animals likewise manifested hepatic ATP levels comparable to those of the age-matched controls. The ATP data in all Gunn rats agree closely with those obtained in normal Wistar rats of the same age (Schenker and O'Donnell, 1965).

DISCUSSION

Previous investigations *in vitro* have shown conclusively that unconjugated bilirubin depresses phosphorylation and, to a lesser extent, respiration of liver mitochondria (Ernster, 1961) and the latter effect has also been shown in liver homogenates in the present study. By contrast, hyperbilirubinaemic liver of newborn guinea-pigs and adult Gunn rats infused with unconjugated bilirubin and of Gunn rats with endogenous hyperbilirubinaemia have been shown in this investigation to sustain normal oxygen consumption in newborn guinea-pigs (Table II)

TABLE IV

EFFECT OF ENDOGENOUS UNCONJUGATED HYPERBILIRUBINAEMIA ON HEPATIC ATP OF GUNN RATS

Litter No.	Animal Age (days)	Total Serum Bilirubin ¹ (mg./100 ml.)	Hepatic ATP Levels			
			Gunn Rats		Control ²	
			μM/g. Liver (wet weight)	μM/g. Liver Protein	μM/g. Liver (wet weight)	μM/g. Liver Protein
1	2	2.3 ^a	3.45	25.54	3.09	18.30
					3.16	18.32
			3.85	25.42	3.93	24.54
2	6	2.0 ^a	3.11	20.82	3.09	22.25
			3.30	23.50	2.56	23.58
3	9	9.0	2.96	17.33	2.82	20.21
4	25	5.5	3.22	17.02	3.18	20.51
					3.37	22.87
5	Adult	7.0	3.99	19.91	4.08	19.20
		4.5	3.93	21.73		
	Mean	5.1	3.48	21.41	3.25	21.09
	± S.D.		± 0.38	± 3.08	± 0.34	± 2.18

¹No direct-reacting bilirubin was detected.²Controls were heterozygous litter mates without hyperbilirubinaemia.^aSpecimens pooled from these animals.

and normal (control) ATP levels in all three groups of animals (Tables I, IV). Thus there was a difference in the effects *in vivo* and *in vitro* of unconjugated bilirubin on liver, although the effects *in vitro* were noted at lower bilirubin levels (12 mg./100 ml. medium) and during a shorter time (10-15 minutes) (Ernster, 1961) than were employed in most of the present studies. These differences could be due both to a higher concentration of the pigment in hepatic mitochondria in the experiments *in vitro*, or to the retention of some protective substance(s) or compensatory mechanism for phosphorylation in the living animal. The intrahepatic concentration *in vivo* of labelled bilirubin in the present study (Table III) was similar in serum and in the liver homogenate-bilirubin incubation media. This essentially equal liver/serum bilirubin concentration gradient agrees with results obtained in adult Gunn rats (Schmid and Hammaker, 1963) and in normal rats infused with Tm-exceeding loads of unconjugated bilirubin (Billing, Maggiore, and Cartter, 1963). In normal adult rats given smaller loads of tritiated bilirubin a higher concentration of the labelled pigment was found in liver as a whole, but only 5.5% of total hepatic isotope was recovered from lysosome-free mitochondria which normally constitute about 18% of liver weight (Brown *et al.*, 1964). Since, however, a similarly low intramitochondrial concentration of the pigment was found in liver homogenates *in vitro* after addition of a comparable quantity of bilirubin (Brown *et al.*, 1964), these facts, which do not discriminate between unconjugated and conjugated bilirubin, may not explain our results. No comparable assessments have been made of the unconjugated fraction in lysosome-free liver mitochondria of newborn animals given large pigment loads or of liver homogenates *in vitro*, employing high bilirubin concentrations. Therefore the first postulate, though a likely one, remains unproven. Regarding the second possibility, albumin and various inorganic ions *in vitro* inhibit the cellular toxic effect of unconjugated bilirubin (Ernster, 1961). The role of such factor(s) or of possible compensatory mechanism(s) for generating high energy phosphates *in vivo*, in the presence of high concentrations of unconjugated bilirubin, is entirely speculative.

Nevertheless it is evident from this study that unconjugated hyperbilirubinaemia, comparable in degree and duration to that employed *in vitro*, did not impair the hepatic respiratory mechanism or phosphorylation in viable animals. In addition, prolonged lower-grade hyperbilirubinaemia similar to that observed in many human diseases likewise failed to affect hepatic ATP levels, in contrast to kernicteric Gunn rat brain wherein we have recently noted a decreased ATP concentration in those areas which were hyper-

pigmented and appeared to be functionally damaged (Schenker, McCandless, and Wittgenstein, 1964). Comparison of respective susceptibility of liver and brain *in vivo* to the cytotoxic effects of unconjugated bilirubin is hampered by the difference in the experimental design for the two studies and lack of accurate measurements of bilirubin concentration in kernicteric brain. Visual estimation of bilirubin content in kernicteric Gunn rat brain, coupled with extraction of its pigment with chloroform in preliminary studies, and the finding of low cerebral bilirubin levels in newborn puppies infused with lethal loads of unconjugated bilirubin (Waters, 1961), suggest that the decline in ATP in hyperpigmented kernicteric brain occurs at bilirubin levels lower than those found innocuous for the liver. This apparent susceptibility of the brain to unconjugated bilirubin, for which no specific explanation is available, is also seen in the differential effect of various anaesthetic agents on cerebral and hepatic respiration (Hoeck, Matteo, Fink, and Hoskins, 1965). It has recently been suggested that the selective toxicity of bilirubin for the central nervous system *in vivo* may be related to the less effective protective effect of albumin at that site (Menkin, Barrett, Swarm, and Berlin 1966).

Two comments are pertinent to the interpretation of the hepatic ATP data presented. (1) Hepatic phosphorylation in this study was assessed from measurement of an ATP concentration representing the result of formation and utilization of the nucleotide. The normal hepatic respiration and structure (light microscopy) in the bilirubin-infused guinea-pigs suggest that the normal nucleotide level observed was not maintained by a compensatory decrease in its utilization. In fact, intrahepatic metabolism of bilirubin, although slow in the newborn guinea-pig (Table III) (Schenker and Schmid, 1964), is believed to be an energy-requiring process (Combes, 1964; Dutton, 1961) and theoretically should augment ATP consumption. It is, therefore, especially noteworthy that even under these circumstances, the newborn animals, with a limited complement of enzymes required for oxidative phosphorylation (Dallman and Schwartz, 1964), were able to maintain control levels of the nucleotide. (2) Hepatic ATP *in vivo* is generated in mitochondria which are evenly distributed in liver (Lehninger, 1964), and sampling of various parts of liver, indeed, gives reproducible ATP values (Schenker and O'Donnell, 1965). This technique, however, does not preclude the possibility that a small, but biologically important, structural or functional intramitochondrial pool of the nucleotide may be selectively depleted by the unconjugated bilirubin, although this hypothesis is not supported by the findings of normal

hepatic function, except for bilirubin metabolism, in certain states of benign prolonged high-grade unconjugated hyperbilirubinaemia (Schmid, 1960).

SUMMARY

It has been suggested on the basis of studies *in vitro* that unconjugated bilirubin in high concentrations is cytotoxic by uncoupling oxidative phosphorylation, thus interfering with the intracellular synthesis of adenosine triphosphate. In the present study, this concept has been tested *in vivo* for liver by measuring hepatic ATP concentration and respiration in animals with high-grade unconjugated hyperbilirubinaemia. At plasma levels of unconjugated bilirubin, similar in magnitude and duration to those employed in studies *in vitro*, no significant effects on hepatic ATP or respiration were observed *in vivo*. The present data suggest that unconjugated hyperbilirubinaemia observed in human disease states does not interfere with hepatic oxidative phosphorylation.

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