proteins rather than in small molecules like glutathione may provide the reactants in the case of mitochondrial phosphorylation. It is not without interest that a thiol and a disulfide have to be brought together for the system to be coupled. On the basis of this hypothesis the current concepts about conformational and configurational prerequisites for a mitochondrion to show coupled phosphorylation are not difficult to visualize as being part of the picture. The thiol and disulfide might be located in a single protein which has to assume the correct conformation, or they might be in two separate proteins which must come together. These alternatives do not exhaust the possibilities for a similar mechanism in mitochondria. The multiple sulfide-thiol relationships in the nonheme iron proteins may be of special significance for this mechanism of oxidative phosphorylation. The possible role of these proteins in energy conservation has been studied and discussed (*7*).

The reaction described here differs from model reactions with oxidation of thioethers in anhydrous pyridine (*4*, *5*) in that it uses only common biological materials, occurs in an aqueous medium, shows sensitivity to catalytic amounts of uncouplers, and can function like a DNP-dependent adenosine triphosphatase.

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Transfer of Bilirubin Uridine Diphosphate–Glucuronyltransferase to Enzyme-Deficient Rats

Abstract. *Cells from a clonal strain (MH_1C_1) of rat hepatoma were transplanted subcutaneously into two homozygous Gunn rats, which are jaundiced because the enzyme bilirubin uridine diphosphate–glucuronyltransferase is absent from the liver. Because of the enzyme activity present in the transplanted cells, the recipient rats developed the capacity to conjugate bilirubin and reverted in large part to a normal pattern of bilirubin excretion.*

Of the therapeutic approaches to diseases due to enzyme deficiency, induction or transfer of enzyme activity is in many ways the most attractive. We have examined the effect of transplanting a clonal strain (MH_1C_1) of rat hepatoma cells (*1*) into homozygous Gunn rats, mutant Wistar rats which are unable to conjugate bilirubin because they lack bilirubin uridine diphosphate (UDP)–glucuronyltransferase (E.C.2.4.1.17) (*2*). The MH_1C_1 cells contain this enzyme and are capable of conjugating bilirubin in vitro (*3*).

Approximately 15 mg of MH_1C_1 cells from 10-day-old subcultures (*1*) were injected subcutaneously into the flanks of four male Gunn rats (3 weeks old). Tumors were detected in two animals about 3 weeks after transplantation. One week later the common bile duct of one of these rats was cannulated, and blood was removed from the tail for measurement of bilirubin concentration in the serum (*4*). Bile samples were collected in the dark over ice during three consecutive 12-hour intervals for measurement of bile volume and bilirubin concentration (*4*) and for analysis by thin-layer chromatography (*3*). Similar studies were performed in the second rat 8 weeks after transplantation, but in this animal pigment excretion was evaluated further with a tracer dose of [^{14}C]bilirubin and later by infusion of a bilirubin load. Normal rat serum containing 0.05 μc of unconjugated [^{14}C]bilirubin (*5*) (specific activity, 6.4 μc/μmole) was injected intravenously, and frequent bile samples were collected over the next 22 hours. Radioactivity in measured volumes of bile was assayed in a liquid scintillation spectrometer (*5*). The remaining volumes were then combined, and the fraction of the total radioactivity present as [^{14}C]bilirubin was determined by crystallization and radioassay of bilirubin (*5*, *6*) after addition of normal rat bile containing a measured quantity of nonradioactive pigment. Maximum bilirubin excretion was measured (*7*, *8*) during intravenous infusion of unconjugated bilirubin (90 μg per minute per 100 g of body weight); bile samples were collected every 10 minutes for 70 minutes, which was the duration of infusion.

Serum bilirubin concentrations in the two tumor-bearing animals were lower than those of any of five control Gunn rats that had not received MH_1C_1 cells (Table 1). Correspondingly, the biliary excretion of bilirubin in these two animals was substantially higher than that of control Gunn rats, but was lower than that of normal Sprague-Dawley rats. The absorption spectrum of diazo-

Table 1. Bilirubin excretion in normal Sprague-Dawley rats, untreated Gunn rats, and Gunn rats bearing MH_1C_1 tumors. Conjugated bilirubin (pigment I and II) was present in the bile of the normal rats and of both tumor-bearing rats but was absent from the bile of the untreated Gunn rats. The numbers in parentheses indicate the number of rats used. Values given are mean ± S.D.

Rats	Serum bilirubin (mg/100 ml)	Bile bilirubin			[^{14}C]Bilirubin excretion (% bile counts as bilirubin)	Maximum bilirubin excretion	
		Concentration (mg/100 ml)	Rate (μg hr^{-1} 100 g body weight^{-1})	Conjugated (%) *		Rate (μg min^{-1} 100 g body weight^{-1})	Conjugated (%) *
Normal (5)	0†	9.4 ± 1.6	30.5 ± 5.0	76.7 ± 16.3	77.5 ± 7.6	58.3 ± 7.4	82.1 ± 33.9
Untreated Gunn (5)	6.5 ± 1.4	0.7 ± 0.2	1.5 ± 0.9	0	20.7 ± 3.4	0.35 ± 0.2	0
Tumor-bearing Gunn rat 1	2.3	2.4	5.4	44.4			
Tumor-bearing Gunn rat 2	3.0	3.9	11.7	73.0	39.0	2.3‡	41.2

* Based on optical densities of eluted bands of conjugated and unconjugated azobilirubin. † Too low to measure. ‡ Still rising at 70 minutes.

Table 2. Bilirubin UDP-glucuronyltransferase activities in liver and tumor. Values are mean ± S.D. The numbers in parentheses indicate the number of rats used.

Subject	Enzyme activity			
	Liver		Tumor	
	$\mu g\ hr^{-1}$ mg protein^{-1}	mg hr^{-1} whole liver^{-1}	$\mu g\ hr^{-1}$ mg protein^{-1}	mg hr^{-1} whole tumor^{-1}
Sprague-Dawley rats (5)	5.6 ± 2.1	13.9 ± 0.5		
Untreated Gunn rats (5)	0	0		
Tumor-bearing Gunn rat 1	0	0	2.1	0.2
Tumor-bearing Gunn rat 2	0	0	1.6	3.3

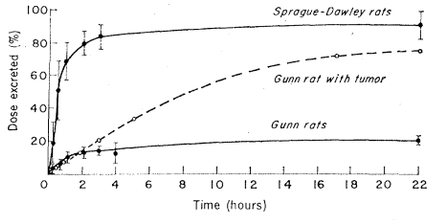

Fig. 1. Cumulative excretion of radioactivity in bile after intravenous injection of [^{14}C]bilirubin in tumor-bearing Gunn rat 2, five untreated Gunn rats, and five normal animals. The fractions of the total radioactivity present as [^{14}C]bilirubin are given in Table 1. Values are mean ± S.D.

tized bile (*4*) from the tumor-bearing rats revealed a major peak at 550 nm, identical to that found in normal rat bile and characteristic of azobilirubin. There was also a smaller, diazo-negative peak at 402 nm, which was not present in bile from normal rats. Bile from untreated Gunn rats contained only small quantities of bilirubin, but relatively large amounts of the material absorbing at 402 nm; the latter probably represents the catabolic pigment observed in Gunn rat bile by Schmid and Hammaker (*9*).

Conjugated bilirubin (pigment I and II) (*10*) was readily identified in the bile of both tumor-bearing rats by thin-layer chromatography (*3*), but was absent from the bile of untreated Gunn rats (Table 1). The diazotized bile pigments formed by reaction with 2,4-dichloroaniline (*11*) were isolated in a second thin-layer system (*3*), which is more reliable for quantitation. Bands migrating as the diazo derivatives of both conjugated and unconjugated bilirubin were found in bile from the tumor-bearing rats; only the unconjugated product was isolated from the bile of untreated Gunn rats (Table 1).

In the tracer study (Fig. 1) the tumor-bearing rat excreted 75 percent of the radioactivity administered as [^{14}C]-bilirubin in the bile in 22 hours, as compared to mean values of 20 percent for untreated Gunn rats and 91 percent for normal animals; however, the rate of excretion was much slower in the tumor-bearing rat than in the normal animals. Thirty-nine percent of the radioactivity excreted in the bile of the tumor-bearing rat was recovered as [^{14}C]bilirubin, a value intermediate between that of untreated Gunn rats and normal controls (Table 1).

Maximum bilirubin excretion was higher in the tumor-bearing rat than in any of the control Gunn rats, but was far below the values observed in normal animals (Table 1). However, pigment excretion in this animal was still rising when the infusion was terminated at 70 minutes, whereas in both control groups it had reached a plateau by 20 to 40 minutes.

At autopsy 0.9 g of partially necrotic tumor tissue was recovered from the first rat; 18.6 g was recovered from the second. Small metastases were found in the mesentery and right kidney of the second animal. Bilirubin glucuronyltransferase activity (*12*) was undetectable in the livers of both untreated and tumor-bearing Gunn rats (Table 2). Enzyme activity was present in the tumors of both experimental animals, although the values were somewhat lower than in MH_1C_1 cells in culture (*3*).

These findings demonstrate that the MH_1C_1 cells conferred a significant capacity to conjugate bilirubin on animals otherwise incapable of performing this function. In studies in vitro (*3*) we have shown that MH_1C_1 cells take up free bilirubin, conjugate it, and then excrete the conjugated pigment back into the culture medium. Clearly, the cells retained these functions after subcutaneous transplantation and simulated the normal pathway for bilirubin excretion in the recipient Gunn rats. In the final step in vivo, the pigment conjugated by the tumor cells was excreted into the bile by the livers of the recipient animals; earlier studies have shown that Gunn rats are able to excrete previously conjugated bilirubin at a normal rate (*13*).

Transplantation of MH_1C_1 cells did not lead to normal levels of bilirubin conjugation, as reflected by the persistence of hyperbilirubinemia, by the slow excretion of [^{14}C]bilirubin, and by the small rise in bilirubin excretion in the infusion experiment. This is not surprising in view of the limited total enzyme activity and the absence of a biliary system in the tumors. In addition, the retention in the bile of the material absorbing at 402 nm and the persistent excretion of a large fraction of injected [^{14}C]bilirubin as labeled nonbilirubin products reflect the continued operation of the alternate metabolic pathways by which Gunn rats usually excrete bile pigment (*9*).

The human counterpart of the Gunn rat, the child with the Crigler-Najjar syndrome (*2*), is at grave risk because of severe unconjugated hyperbilirubinemia. This condition is not amenable to therapy with barbiturates (*8*, *14*), although phototherapy may prove to be of value (*15*). Transplantation of functional neoplastic tissue holds little promise for direct clinical application, but represents a unique biological model with potential implications for the therapy of this and other inborn errors of metabolism.

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Substrate-Induced Conjugation of Bilirubin in Genetically Deficient Newborn Rats

Abstract. *Bilirubin appears to induce its own conjugation with glucuronide. Bilibrubin uridine diphosphate–glucuronyltransferase activity is relatively high at birth in heterozygous offspring of permanently jaundiced female rats. The postnatal development of the transferase is accelerated in hyperbilirubinemic heterozygotes.*

Human infants, unlike newborn rats, frequently develop neonatal jaundice which clears within a few days even in relatively immature newborns. "Physiological" hyperbilirubinemia of the newborn is thought to reflect incomplete development of the enzyme-catalyzing bilirubin conjugation—bilirubin uridine diphosphate–glucuronyltransferase (E.C. 2.4.1.17) (BGT) (*1*). The presence of direct-reacting (conjugated) bilirubin in amniotic fluid (*2*) and in serums of severely jaundiced infants (*3*) and the experimental stimulation of bilirubin conjugation in utero with pulse injections of the pigment (*4*) suggest that substrate induction may play a role in the perinatal development of the bilirubin conjugating system. This hypothesis has been substantiated with the use of a naturally occurring animal model.

Postnatal BGT activity was studied in heterozygous offspring of a mutant Wistar strain of rats (Gunn) with an autosomal recessive defect in bilirubin conjugation (*5*). Homozygous animals of this strain exhibit permanent unconjugated hyperbilirubinemia caused by deficiency of BGT activity. Jaundiced Gunn rats were mated with normal Wistar rats to produce two types of heterozygotes capable of conjugating bilirubin: type 1, delivered by jaundiced females bred to normal males; and type 2, produced by anicteric normal females bred to jaundiced males. The two types were identical in all respects except for continuous exposure of type 1 fetuses to high concentrations of bilirubin crossing the placenta from the jaundiced mother (*6*).

Newborn litters of both types of heterozygotes and control litters of Wistar rats were divided in groups of three to five animals. Animals were weighed, and runts were excluded. Animals within each group were killed by decapitation at birth or at specific intervals afterward, and blood was collected for serum bilirubin determinations. The liver was homogenized in nine volumes of ice-cold 0.25*M* sucrose containing 1 m*M* ethylenediaminetetraacetate (disodium salt), *p*H 7.4. The relative BGT activity in homogenates was assayed with a micromodification (*7*) of the incubation procedure of Van Roy and Heirwegh (*8*) which measures bilirubin monoglucuronide formation. Serum bilirubin was determined by the Malloy-Evelyn procedure (*9*).

In normal rats, BGT activity approximated 30 percent of adult amounts at birth (Table 1). The capacity for conjugation began to increase from 3 to 6 hours after birth, reached adult values between days 2 and 3, and exceeded adult rates in 4- to 7-day-old animals. Weanlings conjugated bilirubin less actively than mature rats did.

The BGT activity in type 1 heterozygotes was equivalent to that in normal animals at birth with little further development during the first 24 hours. In contrast, type 2 heterozygotes converted bilirubin at half the normal rates at birth, but BGT activity began to increase within 6 hours. Conjugation in both types of heterozygotes developed more rapidly than in normal rats after day 1. As a result of accelerated development, conjugation rates in partially deficient heterozygotes approached normal rats on days 2 and 3. A decline from these rates began on day 5, reducing BGT activity to 50 percent of normal in weanling and mature heterozygotes.

The serum of normal rats and of mature heterozygous Gunn rats was anicteric. Normal newborns occasionally ex-

Table 1. Bilirubin UDP-glucuronlytransferase activity (expressed as the number of micrograms of bilirubin conjugated per gram of liver per 30 minutes). Homozygous Gunn rats had no detectable activity. The numbers in parentheses are the number of animals used. Values are the mean ± S.D.

Age (hours)	Bilirubin UDP-glucuronyltransferase activity		
	Normal rats	Heterozygous rats	
		Type 1	Type 2
0	355 ± 15 (25)	385 ± 18 (20)	145 ± 12 (20)
3	342 ± 18 (15)	367 ± 17 (20)	152 ± 14 (20)
6	344 ± 22 (20)	344 ± 15 (20)	200 ± 13 (25)
12	470 ± 33 (25)	352 ± 12 (25)	240 ± 16 (20)
24	664 ± 47 (20)	421 ± 24 (25)	413 ± 28 (30)
48	943 ± 54 (25)	825 ± 38 (25)	800 ± 47 (25)
72	1120 ± 109 (25)	1030 ± 89 (25)	1070 ± 98 (25)
96	1280 ± 113 (20)	1054 ± 95 (25)	1090 ± 102 (25)
120	1360 ± 120 (20)	1000 ± 103 (20)	980 ± 68 (25)
21 days	1035 ± 60 (15)	488 ± 54 (20)	500 ± 60 (20)
1 year	1147 ± 135 (10)	630 ± 70 (7)	580 ± 65 (10)

Table 2. Serum bilirubin concentration in rats (expressed as the number of milligrams per 100 ml). Values are mean ± S.D. Not detectable, n.d.

Age (hours)	Normal rats	Heterozygous rats		Homozygous Gunn rats
		Type 1	Type 2	
0	0.1 ± 0.1	1.4 ± 0.3	0.1 ± 0.1	1.2 ± 0.8
3	0.1 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	1.4 ± 0.9
6	n.d.	0.1 ± 0.1	0.8 ± 0.2	3.0 ± 1.0
12	n.d.	n.d.	1.0 ± 0.2	4.0 ± 0.5
24	n.d.	0.3 ± 0.2	1.3 ± 0.3	5.0 ± 0.8
48	0.1 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	6.2 ± 1.2
72	n.d.	0.8 ± 0.1	0.8 ± 0.2	6.9 ± 1.5
96	n.d.	0.6 ± 0.2	0.6 ± 0.2	7.8 ± 1.8
120	n.d.	0.4 ± 0.1	0.3 ± 0.1	8.2 ± 1.8
21 days	n.d.	n.d.	n.d.	11.5 ± 2.0
1 year	n.d.	n.d.	n.d.	12.8 ± 2.8