ties of the samples were then compared on the basis of the light emitted when 10 μg of ATP in solution (0.1 ml) were added to 0.3 ml of the sample suspension. Results are given in Table 1.

While the preliminary nature of these observations should be stressed, the protective action of the molecular sieve material, in conjunction with a high vacuum, is readily apparent. It is assumed that the necessity for a high vacuum indicates a requirement for the complete removal of oxygen and water. The higher recovery of activity from the Biogel P-300 relative to Sephadex G-25 can be attributed to the ability of the luciferase molecules with a molecular weight of 100,000 to enter more readily into its larger pores. Total loss of activity after the addition of dextran, serum albumin, and DEAE indicates that the protective effect of the molecular sieve is not due merely to a macromolecule or an electrostatic binding effect. A complete validation of our hypothesis must await further definitive studies, which are now in progress.

An important ramification of these studies is their application to the particular requirements for sterilization of components for planetary experiments (*4*). These preliminary observations may represent a significant step toward sterilization for a biological experiment to be conducted on the surface of Mars.

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Oxidative Phosphorylation in Experimental Bilirubin Encephalopathy

Abstract. *Mitochondria from the whole brain or cerebellum of newborn guinea pigs with experimental bilirubin encephalopathy failed to exhibit uncoupling of oxidative phosphorylation. The pigment concentrations required to initiate uncoupling in vitro are much higher than those found in the brain of neurotoxic animals.*

Experimental work has shown that unconjugated bilirubin interferes with vital cellular functions (*1*), and this toxicity probably is responsible for the encephalopathy encountered in severe unconjugated hyperbilirubinemia of the newborn. Studies of normal liver and brain mitochrondria to which bilirubin was added in vitro suggested that the toxicity of the pigment may result from uncoupling of oxidative phosphorylation (*2*). However, the pigment concentrations required to elicit this phenomenon in vitro are much higher than those found in the central nervous system of animals with experimental bilirubin encephalopathy (*3*). This observation suggests that in the intact brain the neurotoxic effects of the pigment may not be related to the mitochrondrial damage observed under conditions in vitro. To examine this possibility we studied brain mitochondria from newborn guinea pigs after infusion of unbound bilirubin at an hourly rate of 150 μg per gram of body weight for 60 minutes. Under these conditions the animals develop manifestations of bilirubin encephalopathy similar to those in infants with severe hyperbilirubinemia, and the extent of the neurologic injury is related directly to the concentration of pigment in the brain (*3*). The animals were killed 30 minutes or 5 to 16 hours after completion of the infusions, and mitochondria were prepared from the whole brain or from the separated cerebellum (*4*). Untreated animals of the same age served as controls. To the mitochrondrial pellet was added 0.5 ml of suspending solution per gram of brain. The functional stability of the suspensions during and beyond the time of subsequent incubation was ascertained by the absence of mitochondrial swelling in aliquots added to 0.25*M* sucrose in 0.02*M* tris-HCl, *p*H 7.4 (*4*).

Aliquots of the mitochondrial suspensions (0.1 ml) containing approximately 0.4 mg of protein (*5*) were incubated at 32°C in 1 ml of a solution, *p*H 7.4, containing 10 m*M* substrate (either succinate, glutamate, glutamate and 0.1 m*M* 2,4-dinitrophenol, or pyruvate and 1 m*M* L-malate) and 25 m*M* glucose, 25 m*M* sucrose, 0.025 mg of yeast hexokinase (Sigma Type III, practical grade), 5 m*M* adenosine diphosphate, 5 m*M* of $MgCl_2$, 1.25m*M* phosphate, and 20 m*M* tris-HCl. In all experiments oxygen consumption was followed polarographically (*6*) and utilization of inorganic phosphate was determined spectrophotometrically (*7*). Measurements usually were made for 3 to 5 consecutive minutes or until at least 30 percent of the oxygen was utilized (*8*).

The cerebellum is one of the areas of the central nervous system most vulnerable to bilirubin (*9*) and in experimental bilirubin encephalopathy may contain the highest concentration of pigment (*3*). Therefore, mitochondria obtained from the cerebellum would be most likely to exhibit pigment toxicity. We found, however, that on incubation with glutamate, cerebellar mitochondria from neurotoxic animals showed a P/O ratio similar to that of preparations from control animals (Fig. 1). Similar P/O ratios were obtained whether the mitochondria were prepared 30 minutes after completion of the bilirubin infusion or 5 to 16 hours later, at a time when the guinea pigs showed advanced neurotoxicity or were moribund (Fig. 1). Furthermore, mitochondria prepared from the whole brain of neurotoxic animals also showed normal P/O ratios with various substrates as shown by the representative results in Table 1. By electron microscopy (*10*) no structural differences could be detected between brain mitochondia from treated

Table 1. Oxidative phosphorylation in mitochondria from the whole brain of a newborn guinea pig 30 minutes after completion of bilirubin infusion. Control: untreated animal.

Substrate	Δ μmole P_i liter^{-1} sec^{-1}		Δ μatom O_2 liter^{-1} sec^{-1}		P/O ratio	
	Bilirubin	Control	Bilirubin	Control	Bilirubin	Control
Succinate	2.19	1.89	0.87	0.75	2.52	2.52
Glutamate	1.52	1.66	.75	.69	2.11	2.41
Glutamate and 2,4-dinitrophenol	0	0	.92	.75	0	0
Pyruvate and malate	2.11	1.42	.79	.67	2.67	2.12

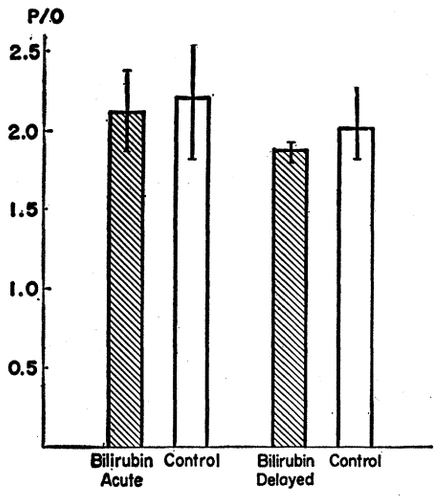

Fig. 1. Oxidative phosphorylation in cerebellar mitochondria of six newborn guinea pigs infused with bilirubin (shaded bars) and of their untreated controls (open bars). The interval between completion of the bilirubin infusion and preparation of the mitochondria was 30 minutes (acute) or 5 to 16 hours (delayed). Results are given as average and range obtained in each group of three animals. In all 12 animals, the average rate of oxygen consumption was 1.42 (S.D. ± 0.41) μatom liter^{-1} sec^{-1} and the average rate of inorganic phosphate consumption was 2.97 (S.D. ± 1.15) μmole liter^{-1} sec^{-1}.

and control animals. In both groups of guinea pigs, however, virtually complete uncoupling of oxidative phosphorylation was obtained when whole brain mitochondria were incubated with glutamate in the presence of 2, 4-dinitrophenol (Table 1).

By contrast, in confirmation of earlier reports (*2*), the addition of bilirubin to brain mitochondria in vitro regularly led to uncoupling of oxidative phosphorylation. With glutamate as substrate the P/O ratio was significantly reduced in the presence of 6.7 μM bilirubin, and at higher concentrations of pigment the ratio approached zero (Fig. 2).

Since the apparent functional integrity of cerebellar and cerebral mitochondria obtained from animals with manifest bilirubin encephalopathy could be caused by loss of pigment during the preparative procedures, tracer methods were used to estimate the subcellular distribution of bilirubin in the brain. Pigment concentration in the whole brain and in the mitochondrial fraction was determined in two newborn guinea pigs after infusion of C^{14}-bilirubin (*3*), and the findings were compared with those obtained by the addition of C^{14}-bilirubin to brain homogenate in vitro. The brains of each of the two infused animals contained 10.6 μg of C^{14}-bilirubin per gram, of which 1.0 and 1.4 percent were recovered in the mitochondria, whereas after the addition of 37.5 μg of C^{14}-bilirubin per gram to the brain homogenate, 3.3 percent of the pigment appeared in the mitochondrial fraction. These values are similar to those recently reported for the subcellular distribution of bilirubin in the liver (*11*). Our findings suggest that only a minute portion of the total pigment in the brain is associated with the mitochondria. Moreover, even if all of the bilirubin in the brain of neurotoxic animals were localized in the mitochondria and none were lost during cell fractionation, the pigment concentration in the incubation mixture still would not exceed 3.6 μM, which is less than the concentration required to initiate uncoupling of phosphorylation in vitro (Fig. 2).

The present observations suggest that uncoupling of oxidative phosphorylation may not be the decisive biochemical event underlying pigment toxicity in experimental bilirubin encephalopathy.

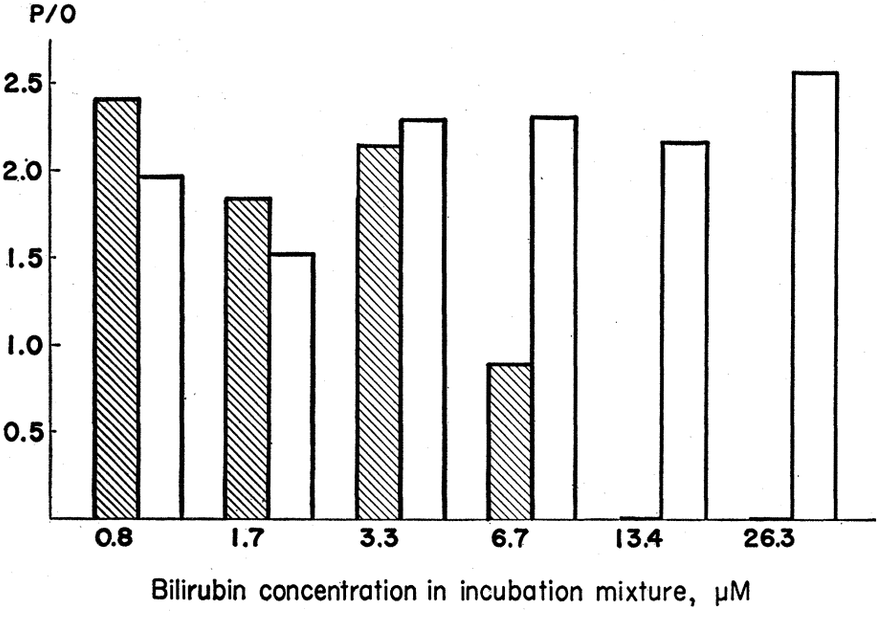

Fig. 2. Oxidative phosphorylation in mitochondria from the whole brain of a newborn guinea pig, to which were added various concentrations of bilirubin in vitro (shaded bars). Control mitochondria were prepared identically but without bilirubin (open bars). Oxygen consumption ranged from 0.95 (S.D. ± 0.06) μatom liter^{-1} sec^{-1} in control and unaffected mitochondria to 0.63 μatom liter^{-1} sec^{-1} with the highest bilirubin concentrations.

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