beam (step c-1 of Fig. 1), and portions were removed after every additional pulse for analysis in the ultracentrifuge. Very similar results were obtained (Fig. 3), except that there remained a small residual denatured peak, apparently due to a small volume of solution which was outside the laser beam during the first pulse, and which therefore received few or no monoadducts.

A decrease in buoyant density of the double-stranded DNA peak with increasing light exposure is apparent in Fig. 2. This is due to the increasing number of adducts in the DNA. The effect is linear, and corresponds to a decrease in density of 0.271 g/cm^3 per AMT/base pair. This compares with a value of 0.182 reported for trimethylpsoralen (*2*). No comparable shift occurred in the series in which the unreacted AMT was removed after one pulse (see Fig. 3).

The psoralen-DNA system may be further characterized by measuring the quantum yields of the first and second photoreactions, and it should be possible to measure the lifetime of the putative triplet intermediate by using two laser pulses separated by an adjustable time delay, to determine the time interval required for the appearance of cross-links. We feel that the ability to separate the two photoreactions in psoralen cross-linking which we have demonstrated here will be especially valuable in applications to living systems.

BRIAN H. JOHNSTON
MARK A. JOHNSON
C. BRADLEY MOORE
JOHN E. HEARST

Department of Chemistry, University of California, Berkeley 94720

References and Notes

1. C.-K. J. Shen and J. E. Hearst, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2649 (1976); *ibid.*, in press.
2. ——, *J. Mol. Biol.*, in press.
3. G. Wiesehahn and J. E. Hearst, in *Molecular Mechanisms in the Control of Gene Expression*, D. P. Nierlich, W. J. Rutter, C. F. Fox, Eds. (Academic Press, New York, 1976), pp. 27–32; C. V. Hanson, C.-K. J. Shen, J. E. Hearst, *Science* **193**, 62 (1976); T. R. Cech and M. L. Pardue, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2644 (1976).
4. P.-S. Song, M. L. Haster, T. A. Moore, W. C. Herndon, *Photochem. Photobiol.* **14**, 521 (1971).
5. S. T. Isaacs, C.-K. J. Shen, J. E. Hearst, H. Rapoport, *Biochemistry*, in press.
6. The proportion of molecules which receive no cross-links is $e^{-\bar{N}}$, where $\bar{N}$ is the average number of cross-links per molecule.
7. E. K. F. Bautz and J. J. Dunn, in *Procedures in Nucleic Acid Research*, G. L. Cantoni and D. R. Davies, Eds. (Harper & Row, New York, 1971), vol. 2, p. 744.
8. We thank M. F. Maestre for the phage samples. Supported by NIH grant GM-11180 and American Cancer Society grant NP-185.

21 April 1977

Reye's Syndrome: Patient Serum Alters Mitochondrial Function and Morphology in vitro

Abstract. *A direct relationship between a putative Reye's syndrome "serum factor" and generalized mitochondrial damage has been demonstrated in vitro. The clinical features of Reye's syndrome may be secondary to disrupted mitochondrial structure and a consequent impairment of energy-linked functions involving many organs.*

Reye's syndrome (RS) is a grave, enigmatic illness of childhood (*1*), often preceded by an otherwise uneventful presumably viral illness (*2*). The course of RS has been subdivided (*3*) into five clinical stages (I to V), beginning with intractable vomiting and lethargy, followed by progressive rapid deterioration of neurological function. Of 349 cases reported in a 1974 epidemic in the United States, only 207 survived (*4*).

Patients with RS have high concentrations of serum ammonia, high serum transaminase activity, and prolonged prothrombin times, which have suggested a hepatic basis for the concomitant encephalopathy (*5*). Morphological studies of liver biopsies from RS patients by Partin *et al.* (*6*) revealed alterations in mitochondrial structure well correlated with the severity of the illness, but the etiology of the mitochondrial damage remained unexplained. The suggestion that an accumulating metabolite or an exogenous toxin in the serum may be a causative factor has been made repeatedly. Using a modification of the *Limulus* assay, Cooperstock *et al.* (*7*) were able to demonstrate endotoxin-like activity in the serum of RS patients, but the relation (if any) of this activity to the cause of the illness was obscure.

In searching for an etiological "serum factor," it seemed logical to test the effect of RS serum directly on mitochondria in vitro. With this approach, it was possible to demonstrate an effect of RS serum on both the respiratory function and the ultrastructure of isolated rat liver mitochondria. These results may provide an advance toward understanding this puzzling illness. Furthermore, the effects of RS serum on animal mitochondria in vitro may be potentially useful in combination with other clinical indicators for diagnosis and evaluation of treatment of RS patients.

The 24 control and RS serums used in this study were obtained from three widely separated geographical locations (*8*) and had been stored at −20° or −70°C for periods varying up to 2 years. All the serums were lyophilized and reconstituted to one-fifth the original volume in distilled water. Microliter portions of the concentrated serums were then tested for an effect on mitochondria in vitro. Nine of the 24 samples were tested without prior knowledge of the clinical state of the donors.

Respiratory activity of isolated rat liver mitochondria (*9*) (1 mg of protein in a 1-ml assay) was assessed polarographically at 30°C by monitoring O_2 consumption with a Clark electrode (Yellow Springs Instruments). The assay medium consisted of 0.225*M* sucrose, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 5 m*M* $MgCl_2$, 15 m*M* KCl, 15 m*M* NaH_2PO_4 buffer, and 50 m*M* tris-HCl, *p*H 7.5. The substrate used was 10 m*M* glutamate. Several parameters of mitochondrial respiration (*10*) were followed, including state 3 and state 4 oxidation rates, ADP/O ratio (ADP, adenosine diphosphate), and respiratory control ratio (RCR), the latter being the most important criterion of functional integrity. The course of a typical assay in the absence of added serum is shown in assay a in Fig. 1. Normal human serum had little effect on respiratory activity (assay b in Fig. 1), but serum from a deeply comatose RS patient (clinical stage IV) doubled the state 4 oxidation rate (assay c in Fig. 1) and consequently reduced the RCR. Changes in other parameters were not as readily apparent although the state 3 oxidation rate usually was increased somewhat in the presence of RS serum.

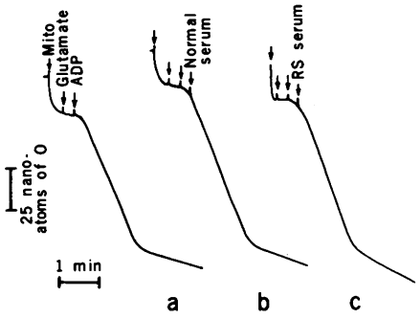
The magnitude of the effect of RS serum on RCR was dependent on the amount of serum added to the assay. Lower RCR's in the presence of RS serum were not restored with 3 percent fat-free bovine serum albumin in the assay, which suggests that the effect was not nominally due to free fatty acids (*11*). The effect of added RS serum could not be reproduced by adding ammonia (50 μl of 5 m*M* NH_4Cl) or salt (10 μl of 5× concentrated 0.9 percent NaCl) solutions to the assay. Nor was the effect diminished by prior deamination of the serum.

Ten microliters of concentrated serum from comatose RS patients (clinical stage III-IV) was sufficient to decrease

RCR's to an average of 49.4 percent of control values (Table 1). Serums from normal donors or from comatose patients with chronic cirrhosis, autoimmune disease (lupus erythematosus), or with chronic active liver disease (microcystic) (*12*) had no effect on mitochondrial function. Serums from RS patients after "total body washout" (*13*) or exchange transfusion, or both (*14*), did not change RCR's (see Table 1). Serum from a patient with salicylate intoxication reduced the RCR to 71 percent of control.

All of the data reported thus far were obtained with rat liver mitochondria, but identical results were seen with liver mitochondria from mouse and guinea pig. Furthermore, RS serum also reduced RCR's in mitochondria isolated from tissue other than liver, namely, rat brain. This was not surprising since many aspects of oxidative metabolism are basically similar in all eukaryotic cells.

In addition to impairing mitochondrial function, RS serum had a striking effect on mitochondrial morphology. In the presence of normal serum, cristae were expanded and aggregated, and the matrix space was condensed (Fig. 2A). This



	a	b	c
State 3	65.3	64.0	72.0
State 4	5.7	6.2	13.6
ADP/O	3.6	3.1	3.3
RCR	11.5	10.3	5.3

Fig. 1. Comparison of the effect of normal serum with that of Reye's syndrome (RS) serum on respiratory function of isolated rat liver mitochondria. Mitochondria were isolated and assayed polarographically (*9*). Potentiometric recordings of O_2 versus time are shown for a typical experiment. In the first assay (a), mitochondria (*mito*) were added, followed by substrate (*glutamate*) and ADP. The same additions were made to assay b, as noted by arrows, except that 10 μl of 5× concentrated normal serum was added at the time indicated. Assay c was the same as b, except that the serum added was from a comatose RS patient. In all the assays, the rapid rate of O_2 utilization after the addition of ADP is state 3 respiration. The slower rate after ADP is depleted is state 4. The numerical values for states 3 and 4 in each assay are given as nanoatoms of O per minute; ADP/O is the amount of ADP added (300 nmole) divided by the amount of oxygen (nanoatoms) used in state 3. Respiratory control ratio (*RCR*) is state 3/state 4, and indicates the degree to which respiration is controlled by ADP.

Table 1. Effect of serums from normal donors or from patients with Reye's syndrome (RS), or other illnesses, on the respiratory control ratio (RCR) of rat liver mitochondria. Experimental conditions are described in the text and in Fig. 1. Data are expressed relative to the RCR obtained with no serum added to the assay (percentage of control RCR). S.D., standard deviation.

Serum donor	*N*	Percentage of control RCR		
		Mean	Range	S.D.
Normal	5	100.0	88 to 111	10.7
RS, stage III-IV	9	49.4	26 to 86	19.8
RS, stage III-IV, after exchange transfusion or total body washout	4	114.5	83 to 164	34.7
Adult grade III-IV hepatic coma (cirrhosis)	3	97.3	86 to 115	15.5
Chronic active liver disease (microcystic)	1	107		
Lupus erythematosus	1	106		
Salicylate intoxication (48 mg/100 ml)	1	71		

"aggregated" configuration is typical of well-coupled mitochondria isolated in sucrose solutions, especially in conditions that stimulate respiration (*15*). Serum from RS patients induced a remarkable change (Fig. 2B). The matrix was rarefied and expanded; most of the cristae had disappeared, but those still visible were greatly contracted, in general resembling the "orthodox" configuration of uncoupled mitochondria (*15*). The morphological changes induced by RS serum in vitro correspond well with those observed clinically in RS liver biopsies (*6*). Ultrastructural studies of RS brain biopsies have revealed similar mitochondrial damage in neurons (*16*).

The source and nature of the RS serum factor remain to be elucidated. Serum samples available for study are limited, but preliminary results suggest a relatively small molecule because an ultrafiltrate (Amicon UM 2 filter, 2000 molecular weight cutoff) of serum was just as effective as whole serum in reducing RCR's. Changes in mitochondrial structure and function like those observed with RS serum can be induced by a variety of agents (*15, 17*).

The demonstration of a direct effect of RS serum on mitochondria should cast serious doubt on the thought that RS is fundamentally a liver disease. It is just as likely that there is broad primary involvement of mitochondria in other body organs. The lower RCR's and the morphological changes induced by RS serum resemble classic "loose coupling" of oxidative phosphorylation. A general impairment of mitochondrial function is consistent with the clinical picture of RS. Organ functions would fail in order of their relative dependence on aerobic energy metabolism and on each other. Predictably, brain is especially susceptible, primarily because this tissue is almost entirely dependent on aerobic metabolism for adequate synthesis of adenosine triphosphate (ATP) and also because of elevated concentrations of ammonia due to simultaneously failing liver function. Brain function declines rapidly in RS and is less readily recovered than liver function, which has an active glycolytic pathway as a back-up to synthesize ATP.

Other clinical features, such as lactic acidosis, elevated free fatty acids, nitrogen wasting, cellular fat accumulation, and hyperammonemia may be explained in the context of primary mitochondrial damage. Ordinarily, any impairment of oxidative phosphorylation will stimulate glycolysis via the Pasteur effect and increase lactate production. In the face of the consequent large increase in glucose utilization, transient hypoglycemia might ensue, followed by fatty acid mobilization and increased protein breakdown. Anaerobic insults are known to cause mitochondrial changes and microvesicular fat accumulation (*18*), similar

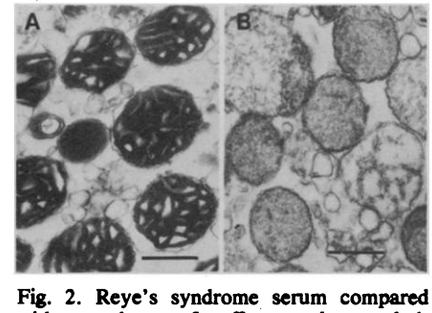


Fig. 2. Reye's syndrome serum compared with normal serum for effects on the morphology of respiring rat liver mitochondria in vitro. Mitochondria were incubated, as described for the polarographic assays in Fig. 1, in the presence of 20 μl of normal serum (A) or 5× concentrated RS serum (B) per milliliter. After 10 minutes at 30°C, preparations were centrifuged at 8000*g*. The pellets were sequentially fixed and stained in Karnofsky's fixative, cacodylate-buffered 1 percent osmium, and 1 percent uranyl acetate. After the pellets were embedded in Epon-Araldite, electron micrographs of thin sections were taken with a Phillips 200 electron microscope. Scale, 0.5 μm.

to those seen in RS. Concentrations of ammonia might be elevated for at least three reasons: urea synthesis is dependent on ATP, two of the urea cycle enzymes are intramitochondrial, and the nitrogen load is augmented because of increased protein catabolism.

The results of this study point to a probable RS serum uncoupling factor other than fatty acids that directly affects mitochondrial organization and function. Since the clinical course of RS is consistent with a general disruption of energy-linked functions that may be primary in several tissues, it is proposed that the as yet unidentified factor may have an important role in the pathogenesis of RS. This hypothesis immediately suggests three corollaries. (i) Since a typical cell may have several thousand mitochondria, a fairly large or very specific insult may be needed to cause serious impairment of energy-linked functions. Any factor that will affect mitochondrial function may thus be synergistically important in the development of this illness. In some cases, salicylates may have such a role, since they are often given during the prodrome illness and since salicylate metabolites are known uncouplers of oxidative phosphorylation (*19*). Only 0.1 percent of children contracting the typical viral prodrome develop RS (*20*), so the possibility of a genetic susceptibility (for example, to the effects or metabolism of uncoupling factors) also must be considered. (ii) A high serum concentration of any cell-permeable uncoupling factor might be expected to initiate the cascade of events leading to an array of symptoms that mimic those seen in RS. In view of this, it should not be surprising that salicylate poisoning per se is often confused with RS (*21*). Similarly, large doses of free fatty acids, which are known to disrupt mitochondrial energy metabolism (*11*), will produce RS-like features in animals (*22*). (iii) Although clinical treatment to remove toxic factors as well as to correct serum ammonia concentrations is indicated, these measures may not result in immediate improvement. Unlike encephalopathies that are secondary to hepatic failure, recovery of the central nervous system from RS will certainly require additional time for mitochondrial regeneration and cell repair.

JUNE R. APRILLE*
Children's Services, Shriners Burns Institute at the Massachusetts General Hospital, and *Department of Pediatrics, Harvard Medical School, Boston 02114*

References and Notes

1. R. D. K. Reye, G. Morgan, J. Baral, *Lancet* **1963-II**, 749 (1963).
2. J. S. Haller, in *Reye's Syndrome*, J. D. Pollack, Ed. (Grune & Stratton, New York, 1974), p. 3.
3. F. H. Lovejoy, A. L. Smith, M. J. Bresman, J. N. Wood, D. I. Victor, P. C. Adams, *Am. J. Dis. Child.* **128**, 36 (1974).
4. L. Corey and R. J. Rubin, in *Reye's Syndrome*, J. D. Pollack, Ed. (Grune & Stratton, New York, 1974), p. 179.
5. P. R. Huttenlocher, *J. Pediatr.* **80**, 845 (1972).
6. J. C. Partin, W. K. Schubert, J. S. Partin, *N. Engl. J. Med.* **285**, 1339 (1971).
7. M. S. Cooperstock, R. P. Tucker, J. V. Baubler, *Lancet* **1975-I**, 1272 (1975).
8. Serums were from Massachusetts General Hospital and the Shriners Burns Institute, Boston; the University of Kansas Medical Center, Kansas City; and Denver General Hospital, Denver, Colo.
9. J. R. Aprille, J. A. Hom, J. Rulfs, *J. Trauma*, **17**, 279 (1977). Briefly, mitochondria were isolated from liver homogenates in 0.225*M* mannitol, 0.075*M* sucrose, 0.1 m*M* EDTA, and 10 m*M* tris-HCl (*p*H 7.5) by centrifuging at 500*g* for 5 minutes; the supernatant was centrifuged at 8000*g* for 10 minutes. The pellet was washed three times and suspended in the same buffer.
10. B. Chance and G. R. Williams, *Nature (London)* **176**, 250 (1955); *J. Biol. Chem.* **217**, 383 (1955).
11. P. Bjorntorp, H. A. Ells, R. H. Bradford, *J. Biol. Chem.* **239**, 339 (1964).
12. W. H. J. Summerskill, *Gastroenterology* **66**, 450 (1974).
13. L. L. Lansky, M. Fixley, D. A. Romig, P. W. Keitges, M. Boggan, R. L. Reis, *Lancet* **1974-II**, 1019 (1974).
14. R. C. Bobo, W. K. Schubert, J. C. Partin, J. S. Partin, *J. Pediatr.* **87**, 881 (1975).
15. D. E. Green and H. Baum, *Energy and the Mitochondrion* (Academic Press, New York, 1970), pp. 195–198.
16. J. C. Partin, J. S. Partin, W. K. Schubert, R. L. McLaurin, *J. Neuropathol. Exp. Neurol.* **34**, 425 (1975).
17. R. J. Kessler, C. A. Tyson, D. E. Green, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3141 (1976); D. R. Hunter, R. A. Haworth, J. H. Southard, *J. Biol. Chem.* **251**, 5069 (1976).
18. I. Boime, E. E. Smith, F. E. Hunter, *Arch. Biochem. Biophys.* **139**, 425 (1970); R. G. Briggs and J. L. Glenn, *Lipids* **11**, 791 (1976).
19. D. M. Woodbury, in *The Pharmacological Basis of Therapeutics*, L. S. Goodman and A. Gilman, Eds. (Macmillan, New York, ed. 3, 1965), p. 322; W. G. Hanstein, *Trends Biochem.* **1**, 65 (1976).
20. D. W. Reynolds *et al.*, *J. Pediatr.* **80**, 429 (1972).
21. R. G. Rosenfeld and M. I. Liebhaber, *Am. J. Dis. Child.* **130**, 295 (1976).
22. A. M. Glasgow and H. P. Chase, *Pediatr. Res.* **9**, 133 (1975); *Biochem. J.* **156**, 301 (1976).
23. I thank J. Rulfs for technical assistance; J. D. Crawford, W. A. Walker, and R. Sanadi for editing and encouragement; L. Lansky, A. Silverman, W. A. Walker, J. Fisher, and J. Escouru for providing serums and clinical reports; and R. Trelstad and B. Feldman for the electron microscopy. A preliminary report of a portion of these results was presented at the annual meeting of the Americal Association for the Study of Liver Diseases [*Gastroenterology* **71**, 894 (abstr.) (1976)]. Supported by HD 08600.
* Present address: Department of Biology, Tufts University, Medford, Mass. 02155.

4 March 1977; revised 22 April 1977

Alcohol Tolerance in a Cholinergic Nerve Terminal: Relation to the Membrane Expansion-Fluidization Theory of Ethanol Action

Abstract. *Phrenic nerve terminals from rats subjected to long-term ethanol treatment were more resistant to ethanol (in vitro) than terminals from sucrose-fed rats, as measured by the effect of ethanol on the frequency of miniature end plate potentials. Long-term ethanol exposure may thus induce the synthesis of more rigid membrane lipids, reducing membrane "fluidizability." This may provide a neurocellular basis for ethanol tolerance and cross-tolerance with anesthetics and barbiturates.*

Although many behavioral and biochemical changes have been found in alcohol-tolerant animals (*1*), the neurocellular basis of alcohol tolerance and dependence is unclear. Since there have been no reports on the possible development of ethanol tolerance in a single nerve cell, we decided to investigate this by using the phrenic nerve-muscle diaphragm preparation.

Of the many direct and acute effects of ethanol and other alcohols on nerve cells (*2*), one of the more sensitive is the enhancement of spontaneous release of acetylcholine from the cholinergic motoneuron terminal (*3, 4*). It has been proposed that the cellular basis of this alcohol-induced enhancement (of neurotransmitter release) stems from the membrane-fluidizing action of the drug (*5*) in association with its membrane-expanding actions (*6*). Our working hypothesis, therefore, was that the motoneuron terminals of animals subjected to long-term ethanol treatment would ultimately become less responsive to ethanol in vitro, on the basis that the "fluidizability" of the terminals would have been altered.

The effect of ethanol on the spontaneous release of acetylcholine was measured by monitoring the frequency of miniature end plate potentials (MEPP's) in the rat phrenic nerve-diaphragm preparation, using standard methods (*3, 4, 7*). The results, shown in Fig. 1, indicate that the phrenic nerve terminals from ethanol-tolerant rats were more resistant to the in vitro application of ethanol than the terminals from sucrose-fed rats. The results are graphed in accordance with the known semilogarithmic relation between ethanol concentration and MEPP frequency (*4*). Quastel *et al.* (*4*) have established that the logarithm of the frequency is linearly related to ethanol concentration; this has been confirmed by others (*3*) as well as by work in this laboratory, which provides the basis for drawing straight lines