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IDENTIFICATION OF THE REYE'S SYNDROME "SERUM FACTOR"[1]

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SUMMARY: Reye's Syndrome serum stimulates O_2 utilization in preparations of isolated rat liver mitochondria and causes mitochondria to swell. The substance responsible for the respiratory activity has been purified by ion exchange chromatography and isolated in one of three ways: crystallization, high voltage electrophoresis, or high pressure liquid chromatography. All three methods yielded active material with identical UV spectra. Further analysis by GC-mass spectrometry and infrared spectroscopy revealed the active substance to be uric acid. Uric acid probably does not cause mitochondrial injury, but it may deserve attention as a possible prognostic marker in Reye's Syndrome.

Serum from patients with Reye's Syndrome has been shown to stimulate oxygen utilization in preparations of isolated rat liver mitochondria (1-4), and to cause mitochondrial swelling (1,3). For lack of a better term, the substance causing increased oxygen consumption has been referred to as a "serum factor". This finding was potentially important for two reasons. First, because mitochondrial injury is characteristic of Reye's Syndrome (5), it seemed possible that the serum factor might have a pathogenic role in the illness. Second, regardless of its pathogenic importance, clinical studies have suggested that the factor might be a useful prognostic marker if it could be assayed simply (4,6). As an essential step toward resolving these issues, we here report the purification and identification of the active factor.

METHODS

Serum fractionation: The starting material was serum obtained during exchange transfusion (courtesy of Dr. John Partin, Children's Research Foundation, Cincinnatti, OH) from children critically ill with Reye's Syndrome. The serum was frozen initially at -70°C, shipped on dry ice, and

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stored at -20°C until used for the separation procedures described below. The general plan for isolation of the active factor was to fractionate serum by ordinary methods, and to test each fraction for bioassay activity after concentrating to a volume approximately 1/10 to 1/15 the original volume of serum used.

Bioassay for serum activity: The bioassay to detect serum factor activity was performed at 30°C in a 1 ml water-jacketed chamber fitted with a Clark O_2 electrode. To 0.95 ml polarographic assay medium (2) was added 1.5 mg mitochondrial protein, 90 nmoles ADP, 1 ug antimycin a, and 10 ul of the concentrated serum or serum fraction. The rate of O_2 consumption before the serum addition was usually zero. The stimulated rate after the addition of the test fraction is reported in units of ng atoms O/min as a measure of activity.

Ultraviolet spectroscopy: Ultraviolet spectra of the purified unknown were obtained on an Aminco DW 2a spectrophotometer by scanning from 200-350 nm in the split beam mode. Spectra were determined under neutral (0.1 M K_2HPO_4-KH_2PO_4, pH 7.0), acidic (0.1 N HCl, pH 1.0), and basic (0.1 N NaOH, pH 12.5) conditions. The sample cuvette contained 10-25 ul of the concentrated isolated material in a 2.5 ml final volume. The reference cuvette contained only the solvent.

Infrared spectroscopy (IR): IR spectra were obtained for KBr micropellets using a beam condenser and a Perkin-Elmer 283B grating instrument.

Mass spectrometry: Electron impact (EI) and field desorption (FD) mass spectra of the underivatized unknown were obtained using a Varian MAT double-focusing instrument fitted with an EI/FI/FD ion source. For the electron impact spectra the conditions were: ionizing voltage, 70 eV; accelerating voltage, 8 KV; ion source temperature, 200°C; direct insertion probe temperature, 320°C. For field desorption, the conditions were: counter electrode voltage, -3 KV; field anode voltage, +8 KV; ion source temperature, 90°C. Samples were applied to activated emitters prepared from 10 um Tungsten wire by dipping the emitter into a solution of the sample in dimethylsulfoxide or 2-(2-amino-ethylamino) ethanol. Desorption was optimal at 26 ma. emitter current.

Gas chromatographic-mass spectrometry: (GC/MS): Analyses were performed using a Perkin-Elmer 990 gas chromatograph interfaced to a Hitachi RMU-6L low resolution mass spectrometer via a glass jet interface. The MS was scanned repetitively every 4 sec. Data acquisition and control were provided by an IBM 1800 computer. A 6' x 1/4" glass column packed with 3% OV-17 on Gas Chrom Q was programmed at 12 or 16°C/min. MS ion source temperature was 210°C; ionizing voltage, 70 eV; accelerating voltage, 2200V.

Chemical derivatizations for GC/MS Analysis: Trimethylsilyl derivatives of the unknown were prepared by heating the sample with Sylon BFT (1% trimethylchlorosilene in n,o-bis-(trimethylsilyl)-trifluoroacetamide, Supelco Chemical Co.) in acetonitrile for two-and-one-half hours at 90°C or 100°C.

RESULTS

Purification of the Serum Factor. Previous work has shown that serum activity is recovered in the ultrafiltrate after high-pressure filtration of whole serum through an Amicon UM 05 filter which retains molecules of

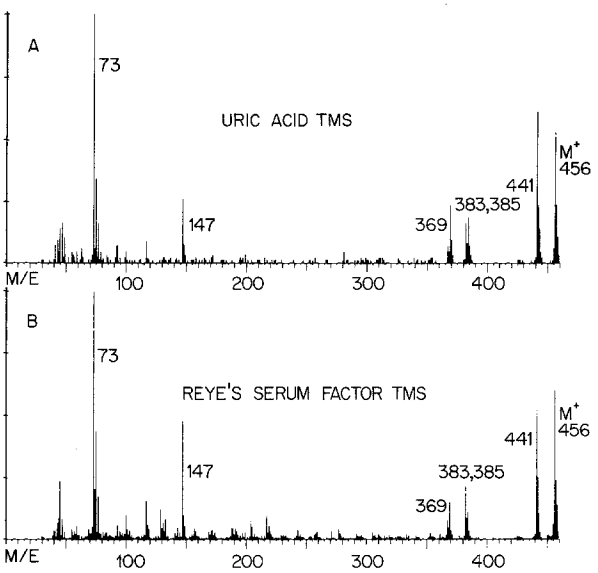

Figure 1. GC/MS results for trimethylsilylated derivatives of (A) uric acid and (B) Reye's serum factor. See text for experimental details.

MW>500-1000 (3). The filtrate was lyophilized and resuspended in a small volume of formic acid, pH 2.8 (.01 N). This was adsorbed to an anion exchange column previously equilibrated in the same buffer. The column was then washed until the effluent was pH 2.8. Formic acid, pH 2.0 (0.5 N), was used to elute the active substance. The active fractions were pooled and applied to a cation exchange column previously equilibrated with pH 2.0 formic acid, and eluted with this same buffer.

Final purification of the active substance was achieved in one of three ways: high voltage paper electrophoresis, high performance liquid chromatography (HPLC), or overnight crystallization from pH 2 formic acid in the cold. All three methods yielded material with identical ultraviolet spectra (see below). The most active fraction from HPLC separation was used for further analysis.

Analyses. GC/MS analysis of the trimethylsilyated unknown revealed the major constituent to be uric acid, present as its tetratrimethylsilyl derivative (Figure 1). GC/MS showed that dioctyl phthalate and 2,6-di-t-butyl-4-methyl phenol (BHT) were both present, but since they did not

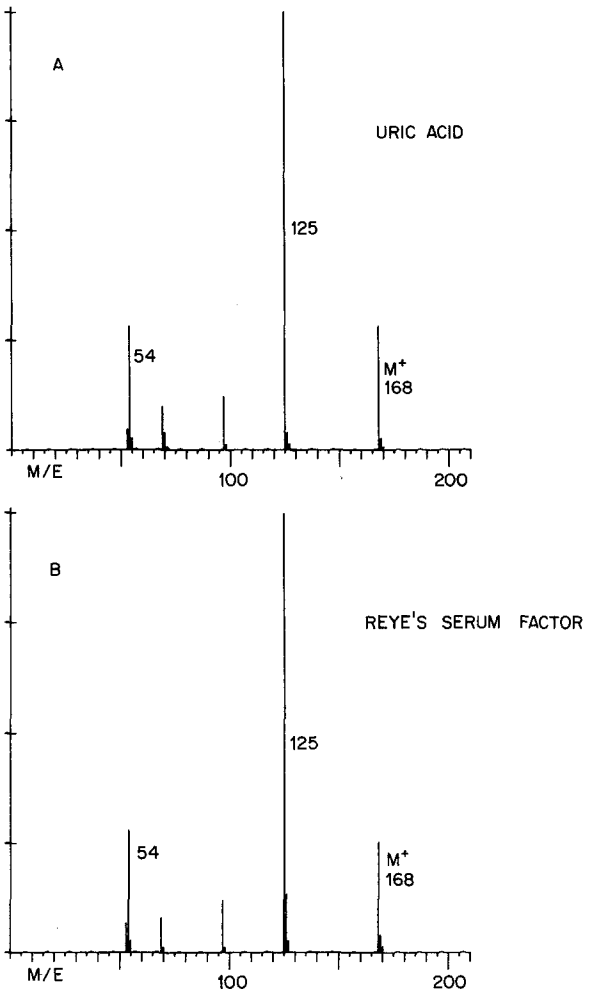

Figure 2. Electron impact mass spectra of (A) uric acid and (B) Reye's serum factor obtained for sample admitted via direct insertion probe.

contribute significantly to the ultraviolet spectrum observed, they were probably only minor contaminants. It was possible to form the TMS derivative of pure uric acid under slightly milder conditions than were required for the isolated unknown, but the difference was minor.

The electron impact (Figure 2) and field desorption mass spectra of the unknown matched those of uric acid taken under the same conditions. The field desorption mass spectra showed a molecular ion cluster and no fragment ions. High resolution measurements of the electron impact mass spectrum was consistent with the spectrum of uric acid [observed m/e

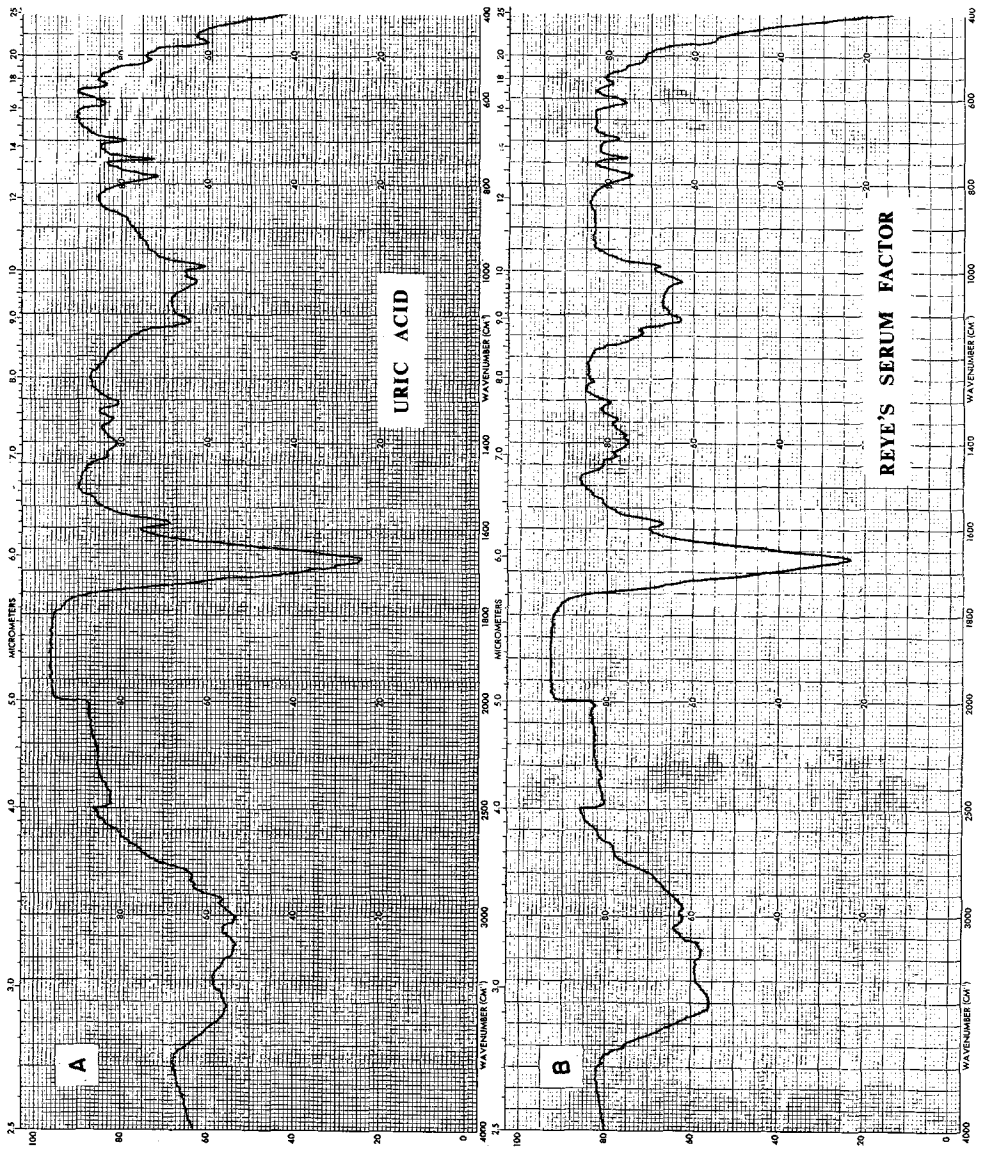

Figure 3. Infrared spectra obtained using a beam condenser for KBr micropellets prepared from (A) uric acid which had been passed through anion and cation exchange columns under conditions used for the isolation of Reye's serum factor and (B) Reye's serum factor after the same purification steps. Scale expansion for (B), 1.5x.

168.0271 ($C_5H_6NO_3$ calc. 168.0283), observed m/e 125.0219 ($C_4H_3N_3O_2$ calc. 125.0225)]. The infrared spectrum of the unknown was also identical to that of uric acid (Figure 3).

The ultraviolet spectrum matched that of uric acid and showed the same dependence upon pH (Figure 4). The O.D. (292 nm) of isolated Reye's serum factor was diminished by uricase at a rate identical to the rate at which an equivalent amount of uric acid was degraded under the same

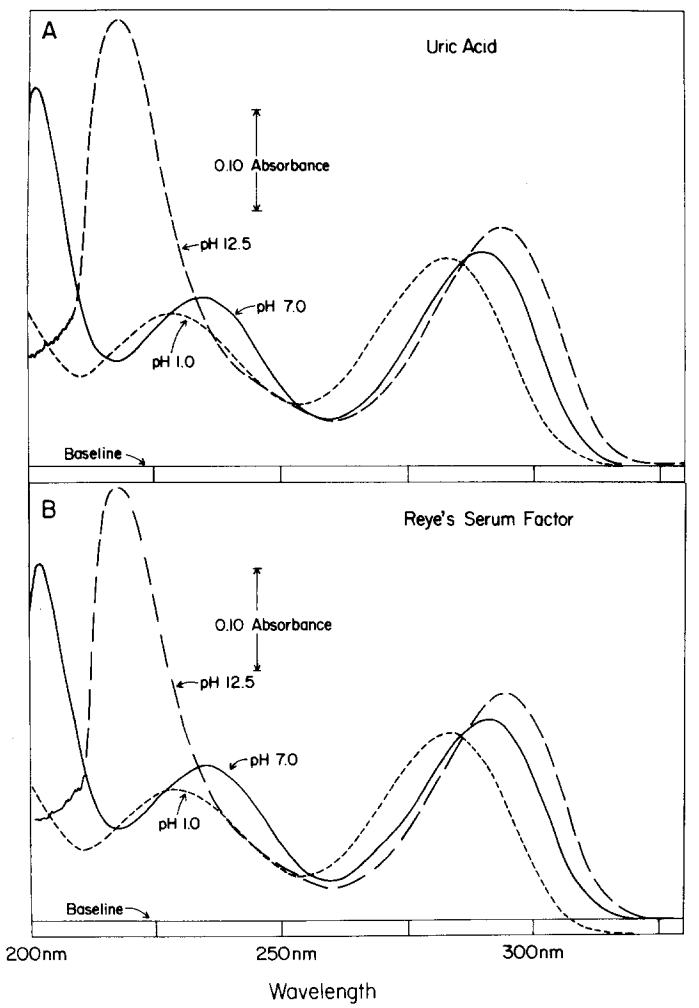

Figure 4. Ultraviolet spectra of (A) uric acid and (B) Reye's serum factor. The spectra were determined by scanning from 200-350 nm at acid (-------), neutral (————), and basic (— — —) pH as described in Methods.

Table 1. Uricase degradation of Reye's factor and uric acid. Reye's serum factor or uric acid was added to 2.5 ml of 60 mM glycine, pH 9.5. At time zero, .01-.02 units of uricase (Sigma Chemical Co., St. Louis, MO) was added and optical density was followed continuously using the dual wavelength pair 292-300 nm. Values are the mean ± S.E. for triplicate determinations.

	O.D. at time 0	% change in O.D. at: 1 min	3 min
Reye's serum factor	.089 ± .003	-17.8 ± 0.3	-47.7 ± 0.2
Uric acid	.087 ± .003	-16.2 ± 0.2	-45.6 ± 0.3

Table 2. Effect of uricase on the bioassay activity of Reye's serum factor and uric acid. Solutions of uric acid and isolated serum factor were adjusted to give similar O.D.'s at 292 nm. Glycine, pH 9.5 was added to a f.c. of 10 mM. Uricase was added and the mixture was incubated at 30°C. Bioassay activity was assayed as described in Methods.

	Activity (ng atoms O/min) initial	uricase + 15 min
Reye's serum factor	19.6 ± 0.9	11.1
Uric acid	20.6 ± 1.1	14.7

conditions (Table 1). Uric acid had bioassay activity identical to that of the serum factor; bioassay activity was decreased after incubation with uricase (Table 2).

DISCUSSION

The substance in Reye's Syndrome sera responsible for the antimycin-a-insensitive stimulation of O_2 consumption in preparations of isolated mitochondria (1-4) has been isolated and identified as uric acid. Identical behavior was observed for the unknown and for uric acid in the separation methods and spectroscopic techniques, indicating that the isolated material is uric acid itself and not a conjugate or other derivative. Uric acid duplicates the stimulation of O_2 consumption but not the mitochondrial swelling caused by whole serum. Whether there is a separate serum "swelling factor" (other than fatty acids, which are known to cause swelling) remains to be seen.

The mechanism by which uric acid stimulates O_2 consumption in the bioassay is not known at this time. Uric acid will reduce cytochrome c (7), which is consistent with the proposed action of the serum factor (2). However, it is more likely that O_2 is used during the oxidation of uric acid by uricase (urate oxidase), a peroxisomal enzyme that may be present in suspensions of rat liver mitochondria prepared by differential centrifugation.

Uricemia has been reported in Reye's Syndrome (8), but the metabolic cause has not been investigated. It does not seem likely that uric acid causes mitochondrial injury in Reye's Syndrome. Both uricemia and mitochondrial dysfunction are probably secondary to some etiological agent(s) If a factor deleterious to mitochondria is present in serum, it was not detected by the bioassay used in this study.

Simple assays are available for the routine determination of uric acid in serum (9). Collected data on "serum factor" activity in serial samples during hospitalization (4,6) show that the factor, now known to be uric acid, remains elevated in fatal cases but decreases in parallel to neurologic improvement in survivors. These data suggest that serial determination of uric acid may be a useful but heretofore overlooked prognostic marker.

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