the third molt, usually by day 20, 3 ml of fresh medium were added at each medium change. No antibiotics were used in the culture media.
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* Present address: Department of Parasitology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, D.C. 20307.
† To whom requests for reprints should be addressed.

6 April 1983

Salicylate and Mitochondrial Injury in Reye's Syndrome

Abstract. *Electron microscopic and spectrophotometric studies showed that salicylate causes gross swelling of mitochondria in isotonic salt solutions. In overall morphology the salicylate-treated mitochondria resembled those from patients with Reye's syndrome. Salicylate analogs such as* m-*hydroxybenzoate,* p-*hydroxybenzoate, and benzoate did not exert this effect. The mitochondria deformed by salicylate tended to return to their original condensed form on removal of the drug.*

Reye's syndrome, an often fatal syndrome of children, is characterized by fatty degeneration of the liver and kidneys, rapid swelling of the brain, seizures, and loss of consciousness. Salicylism (aspirin poisoning) resembles Reye's syndrome in many ways, and it has been suggested that aspirin is somehow linked with the onset of this disorder (*1, 2*).

The mitochondria in Reye's syndrome patients are grossly swollen and deformed. Their matrix is rarefied, the materials in it are coarsely granular, and the cristae are fragmentary, widely spaced, and few in number (*3*). These ultrastructural changes serve as specific criteria in the diagnosis of Reye's syndrome.

A number of agents (inorganic phosphate, thyroxine, glutathione, ascorbate, fatty acids, and Ca^{2+}, among others) (*4*), including many uncouplers of mitochondrial energy transduction (*5*), cause isolated mitochondria to swell in isotonic salt solutions. I now report that salicylate, an uncoupler (*6*), causes a concentration-dependent swelling of osmotically contracted mitochondria in vitro.

Rat liver mitochondria were prepared in accordance with the method of Schnaitman and Greenawalt (*7*), except that bovine serum albumin was excluded from the isolation buffer. Mitochondrial swelling was monitored by measuring changes in the absorbance of light at 565 nm. Figure 1 shows the course of mitochondrial swelling in the presence of salicylate and its close structural analogs. Salicylate caused light absorbance to decrease, denoting swelling, and the rate of the swelling was proportionate to the salicylate concentration (Fig. 1A). Swelling was first noticeable in the presence of 1.6 m*M* salicylate; at concentrations of 16.4 and 32.4 m*M*, the mitochondria reached maximum swelling in 2 and 0.5 minutes, respectively. Rotenone, antimycin A, and oligomycin had no effect on the course of swelling. Swelling also occurred in medium containing KCl or NaCl instead of NH_4Cl, although the rate was considerably lower.

The salicylate analogs benzoate, *m*-hydroxybenzoate, and *p*-hydroxybenzoate did not induce appreciable mitochondrial swelling even at 32.3 m*M* (Fig. 1B). This strongly indicates that the swelling was not due to changes in osmolarity and was unique to salicylate. Addition of 20 μmole of salicylate (final concentration, 6.2 m*M*) to the suspensions containing structural analogs induced rapid swelling. Salicylurate, the major end product of salicylate metabolism in humans (*8*), was without effect.

Mitochondria assumed the typical "condensed form" (*9*) in the isolation buffer with mannitol and sucrose (Fig. 2A). They were contracted, the matrix was electron-dense, the cristae were numerous and well defined, and the intracristal spaces were large. The morphology of these mitochondria is grossly similar to that described by Schnaitman and Greenawalt (*7*), whose isolation procedure was employed. In an isotonic solution of 20 m*M* tris and 130 m*M* NH_4Cl (*p*H 7.4), mitochondria also assumed the condensed form, but exhibited somewhat smaller intracristal spaces and thus a slightly smaller degree of contraction than in the isolation buffer (Fig. 2B).

The ultrastructure of mitochondria in medium containing 20 m*M* tris, 130 m*M* NH_4Cl, and 6.6 m*M* salicylate (Fig. 2C)

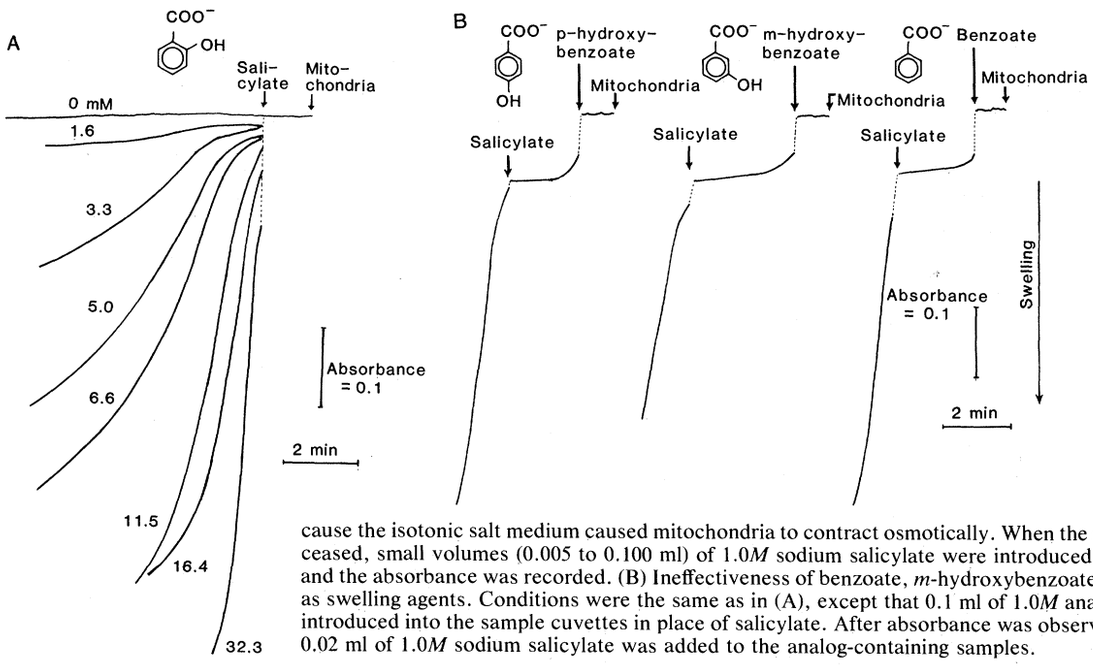

Fig. 1. Effect of salicylate and its analogs on the swelling of rat liver mitochondria. (A) Swelling as a function of salicylate concentration. The sample cuvettes contained mitochondria (1.9 mg of protein per milliliter) suspended in 3.1 ml of 20 m*M* tris and 130 m*M* NH_4Cl (*p*H 7.4). The reference contained the mitochondrial suspension in 20 m*M* tris (*p*H 7.4). The sample suspension gave an absorbance about 0.8 above that of the reference at 565 nm because the isotonic salt medium caused mitochondria to contract osmotically. When the spontaneous swelling had ceased, small volumes (0.005 to 0.100 ml) of 1.0*M* sodium salicylate were introduced into the sample cuvettes and the absorbance was recorded. (B) Ineffectiveness of benzoate, *m*-hydroxybenzoate, and *p*-hydroxybenzoate as swelling agents. Conditions were the same as in (A), except that 0.1 ml of 1.0*M* analog (the sodium salt) was introduced into the sample cuvettes in place of salicylate. After absorbance was observed for at least 2 minutes, 0.02 ml of 1.0*M* sodium salicylate was added to the analog-containing samples.

was drastically different from that of mitochondria suspended in the salicylate-free medium. They swelled to a size several times larger than that seen in the condensed state. The matrix was uniformly dilated and largely electron-transparent; whatever stained materials were present in the matrix were weakly stained, coarsely granular, and dispersed irregularly in patches. The inner membrane was fully stretched and cristae were no longer apparent. As a result of membrane disintegration, some of the mitochondria were at the verge of rupture (arrows in Fig. 2C). None of the mitochondria in this medium exhibited the condensed morphology.

To determine whether the effect of salicylate is reversible, the salicylate-treated mitochondria were washed once with 20 m*M* tris and 130 m*M* NH_4Cl (*p*H 7.4) by centrifugation. Some of the washed mitochondria returned to the condensed form (filled arrows in Fig. 2D), and some of these had very large intermembrane spaces (filled arrowheads), indicating that the outer membrane is less elastic than the inner membrane. Unlike the salicylate-treated mitochondria, even the large mitochondria in the washed preparation (open arrows in Fig. 2D) showed uniform distribution of the coarsely granular materials in the matrix and reappearance of fragmentary cristae (open arrowheads). It should be pointed out that the washed mitochondria had been maximally swollen before the wash (Fig. 1A). Had they been swollen less drastically by a low concentration of salicylate, all of them might have returned to the original morphology.

When the salicylate in the test medium was replaced with *m*-hydroxybenzoate, *p*-hydroxybenzoate, or benzoate, the mitochondria did not swell (Fig. 2, E to G), but looked more or less like those in the salicylate-free salt solution (Fig. 2B).

The electron microscopic results confirm the spectrophotometric finding that salicylate causes mitochondria to swell. This study, however, does not prove that salicylate, which is acquired in the human body as the result of hydrolysis of ingested aspirin, causes mitochondria to swell in situ as well. [Although the therapeutic level of aspirin, 0.5 to 3 m*M* (7 to 40 mg/dl) is, in terms of the salicylate concentration in serum (*10*), high enough to induce swelling, it is unknown what its cytosolic concentration would be at this extracellular concentration.]

Aprille and co-workers (*11*) reported that an unidentified factor of low molecular weight in serum from Reye's syndrome patients caused rat liver mitochondria to swell in vitro. While the morphology of the mitochondria incubated with normal serum was comparable to that shown in Fig. 2A, the mitochondria incubated with patients' serum looked strikingly similar to the salicylate-treated mitochondria (Fig. 2C).

It has been proposed that symptoms of Reye's syndrome are likely if there is an acute insult to the mitochondria (*11, 12*). The fact that salicylate-treated mitochondria and mitochondria from Reye's syndrome patients are ultrastructurally similar adds support to this proposition.

Since salicylate completely abolishes aerobic synthesis of adenosine triphosphate in the mitochondria at a concentration of 1 to 2 m*M* (*6*), it may function as a metabolic poison capable of producing the symptoms of Reye's syndrome. But if aspirin ingestion causes the onset of Reye's syndrome, then why is this disease so uncommon? Under normal physiological conditions, over 80 percent of the salicylate in the human body is converted in liver mitochondria to salicylurate (*8, 13*), which is neither an uncoupler (*6*) nor a swelling agent. If, however, in certain clinical or genetic situations, the metabolism of salicylate is blocked partially or wholly because of a defect in the enzymes involved in this detoxification pathway, then the mitochondria will suffer from uncoupling until the salicylate is disposed of by minor pathways (as through the formation of glucuronides). Finally, other chemical agents capable of uncoupling mitochondrial oxidative phosphorylation (such as some fatty acids and pesticides) may be able to trigger the same symptoms as does salicylate.

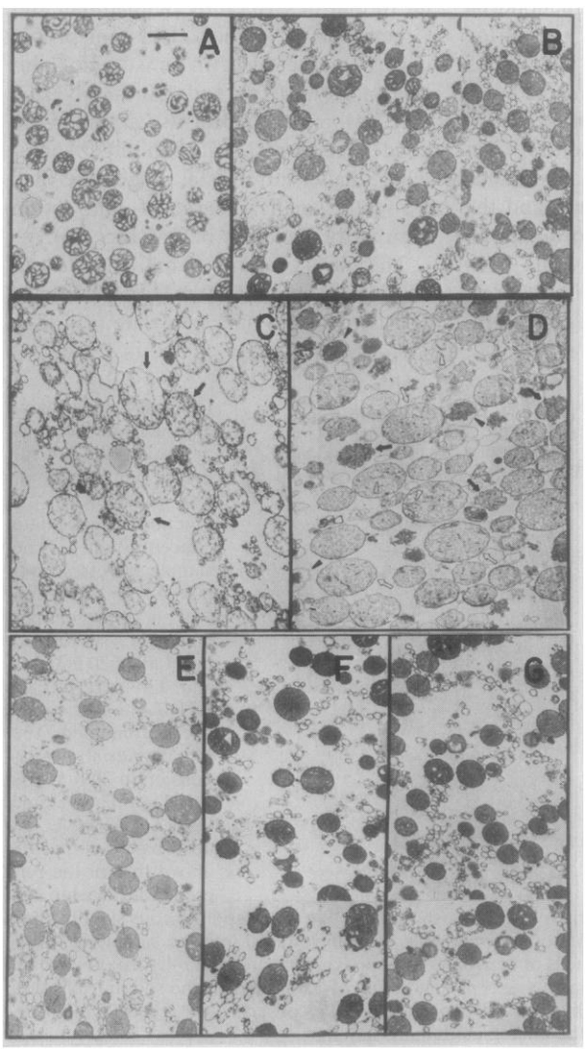

Fig. 2. Electron micrographs of mitochondria in (A) isolation buffer (220 m*M* mannitol, 70 m*M* sucrose, and 2 m*M* Hepes); (B) 20 m*M* tris and 130 m*M* NH_4Cl; (C) 20 m*M* tris, 130 m*M* NH_4Cl, and 6.6 m*M* salicylate; and (D) 20 m*M* tris and 130 m*M* NH_4Cl after treatment with salicylate, followed by washing. Panels E, F, and G show mitochondria that were incubated in the same medium as in C except that *m*-hydroxybenzoate, *p*-hydroxybenzoate, and benzoate replaced salicylate, respectively. Panels B, E, F, and G show two tissue sections each. The concentration of protein in the suspensions was 1.9 mg/ml in all cases. Each incubation was carried out at 23°C for 8 minutes. Mitochondria were harvested by centrifugation, fixed in 2 percent glutaraldehyde, postfixed in 2 percent osmium tetroxide, and then stained with 0.5 percent uranyl acetate. Treated tissues were embedded after dehydration in graded ethanol and cut into silver-gray sections. A JEOL 100C electron microscope was used at 80 kV. Symbols are identified in text. Scale bar, 1 μm.

KWAN-SA YOU
Department of Pediatrics,
Duke University Medical Center,
Durham, North Carolina 27710

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14 March 1983

α-Amanitin Tolerance in Mycophagous *Drosophila*

Abstract. *Six species of* Drosophila *were tested for tolerance to the mushroom toxin α-amanitin, a potent inhibitor of RNA polymerase II. Three nonmycophagous species—D. melanogaster, D. immigrans, and D. pseudoobscura—showed very low survival and long development times in the presence of amanitin. Three mycophagous species—D. putrida, D. recens, and D. tripunctata—showed little or no sensitivity. Analysis in vitro indicated that this tolerance is not based on alteration of the molecular structure of RNA polymerase II.*

Some mushrooms of the genus *Amanita* contain substantial quantities of α-amanitin, a bicyclic octapeptide that is a potent inhibitor of eukaryotic RNA polymerase II, the enzyme which transcribes genes that encode messenger RNA's. Amanitin, therefore, is potentially very toxic to virtually all eukaryotes (*1, 2*). Larvae of wild-type *Drosophila melanogaster*, for example, cannot survive amanitin concentrations greater than about 5 μg/ml in the culture medium. However, amanitin-resistant strains of this species have been selected in the laboratory, and in at least one case (strain C4), the mechanism of resistance resides in an amanitin-insensitive RNA polymerase II (*3*). Although amanitin is considered to be a general toxin, a number of species of *Drosophila* breed, apparently with no ill effect, in various *Amanita* species that may contain up to 5000 μg of amanitin per gram (dry weight) of mushroom tissue, which is about 500 times the median lethal dose (LD_{50}) for wild-type *D. melanogaster* (*4*). The following questions arise: (i) Are mushroom-feeding species of *Drosophila* tolerant of α-amanitin, or do they avoid poisoning by not ingesting amanitin-containing tissues—for example, by selectively feeding on the yeasts and bacteria that grow on deliquescent mushrooms? (ii) If they are tolerant, is this tolerance a consequence of their possessing an amanitin-insensitive RNA polymerase II?

Six species of *Drosophila*, each from a different species group within the genus, were reared in the laboratory on artificial medium containing various concentrations of α-amanitin (*5*). These species included three that rarely, if ever, breed in mushrooms—*D. melanogaster*, *D. immigrans*, and *D. pseudoobscura*—and three—*D. putrida*, *D. recens*, and *D. tripunctata*—whose sole or principal breeding sites are mushrooms, including those that contain amanitin (*6*). For each species we determined, as a function of amanitin concentration in the larval medium, several components of fitness, including egg-to-adult survival, development time, and adult body size (*7*). In addition, RNA polymerase II activity was determined in vitro as a function of amanitin concentration (*8*).

None of the three nonmycophagous species survived amanitin concentrations of 50 μg/ml, with the exception of *D. melanogaster* strain C4, the resistant strain mentioned above that has an al-

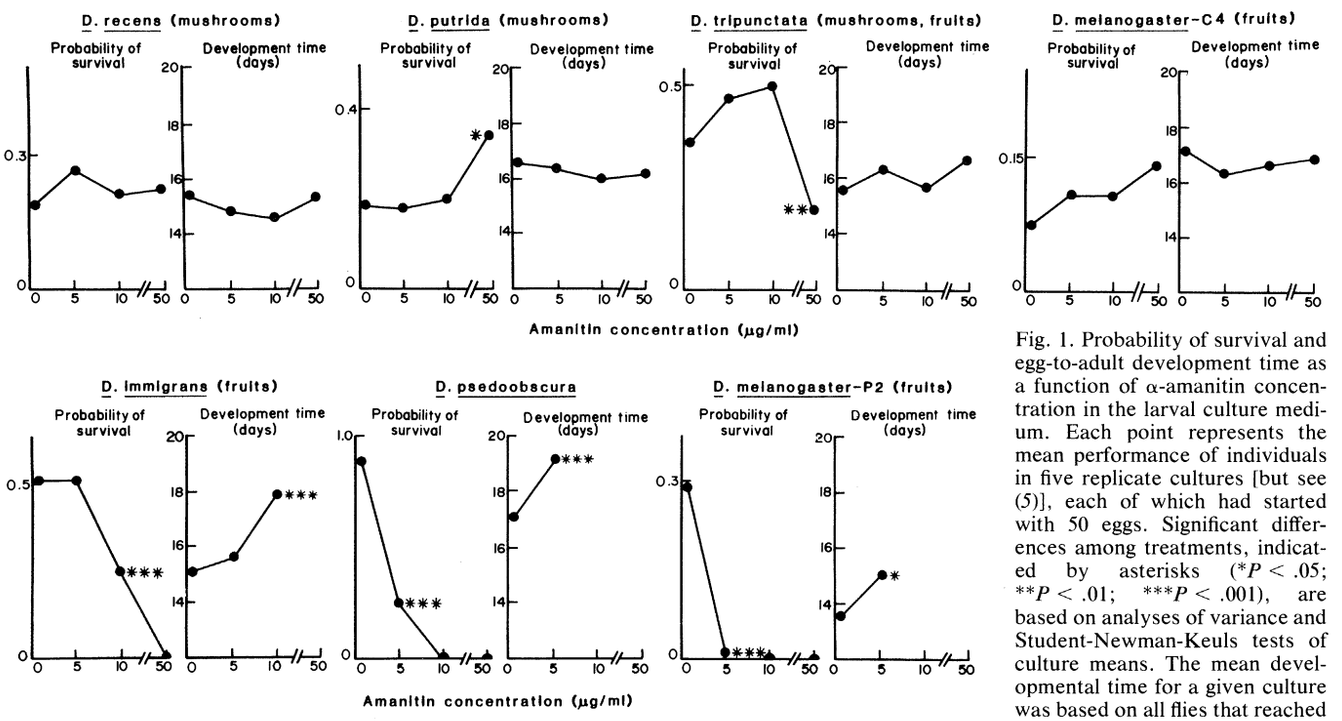

Fig. 1. Probability of survival and egg-to-adult development time as a function of α-amanitin concentration in the larval culture medium. Each point represents the mean performance of individuals in five replicate cultures [but see (*5*)], each of which had started with 50 eggs. Significant differences among treatments, indicated by asterisks (*$P < .05$; **$P < .01$; ***$P < .001$), are based on analyses of variance and Student-Newman-Keuls tests of culture means. The mean developmental time for a given culture was based on all flies that reached adulthood. Points designated by asterisks differ significantly at the level indicated from those representing lower concentrations of amanitin. The principal breeding sites of the flies are indicated in parentheses after the species name; the breeding site of *D. pseudoobscura* is unknown.