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7. The bath contained 50 ml of a physiological salt solution with the following composition (m*M*): NaCl 94, $NaHCO_3$ 24, KCl 5, $MgSO_4$ 1, Na_2HPO_4 1, $CaCl_2$ 2.25, sodium acetate 20, and glucose 10. The solution was equilibrated at 30°C with a gas mixture of 95 percent O_2 and 5 percent CO_2; the *p*H was 7.4.
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10. The difference signal could equally well be plotted downward (a − b) and thought of as "missing light" associated with tension development. Although, as will be apparent from the discussion, the latter approach may prove to have a sounder mechanistic basis, we have adopted the former for the purpose of this presentation because we believe that most readers will be accustomed to thinking of the isometric as the fundamental mode of contraction, and of shortening as a complicating event.
11. Our observation that the time course of the aequorin signal was essentially the same in isometric contractions at two fiber lengths would appear at first glance to be in conflict with a recent report by Allen and Kurihara (*16*), who found that decreases in fiber length tended to prolong the aequorin signal. There is, in fact, no real conflict, for the length changes we have studied were comparatively small, and comparable changes in their experiments produced no more than a 6 to 7 percent change in the duration of the aequorin signal. This change is less than the standard deviation of their measurements, and in the absence of information from greater length changes, would not have been considered significant. We too see a change in the time course of the aequorin signal with greater changes in length. The important point is that changes in the aequorin signal associated with shortening are very much greater than those produced by alterations in length per se.
12. The duration of the plateau of the action potential is known to be longer in isotonic than in isometric contractions (*9*), but since in a given contraction the light of shortening occurs earlier than the change of the action potential, it seems unlikely that the prolongation can be the cause of the change in the Ca^{2+} transient.
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Dipetalonema viteae (Nematoda: Filarioidea): Culture of Third-Stage Larvae to Young Adults in vitro

Abstract. *Infective third-stage larvae of* Dipetalonema viteae *(Nematoda: Filarioidea) were cultured to young adults in a cell-free culture system. Third-stage larvae from the tick vector grew, developed, and molted twice in a medium containing NCTC 135 and Iscove's modified Dulbecco's medium supplemented with fetal bovine serum under a gas phase of 95 percent nitrogen and 5 percent carbon dioxide. The availability of such a culture system for filariids should facilitate studies of their immunology, biochemistry, and sensitivity to drugs.*

Filarial nematodes are important parasites of man and other animals throughout the world; diseases caused by these organisms, such as African river blindness, are especially targeted for study by the World Health Organization (*1*). The culture of filariids in vitro, which thus far has had only limited success (*2*), would be valuable for studies of the immunology and biochemistry of the parasites as well as for the screening of drugs. We now report the culture of a filarial nematode from the third larval stage through two molts to the young adult in a cell-free culture medium. Prior exposure of the larvae to the mammalian host was not required.

We used *Dipetalonema viteae* (Onchocercidae) as a model in developing a culture system for the vertebrate phase of a filariid life cycle, and started with third-stage larvae obtained directly from the arthropod vector. This nematode in jirds (*Meriones unguiculatus*) or hamsters (*Mesocricetus auratus*) has been used in a number of studies on filariasis, including immunological (*3*) and biochemical (*4*) investigations. It has also been used for the screening of potential filaricides in vivo (*5*).

Dipetalonema viteae has a typical filariid life cycle. The arthropod vector, an argasid tick (*Ornithodorus tartakovskyi*), releases third-stage larvae in the vertebrate host while taking a blood meal. These larvae grow and develop in the subcutaneous tissues of the vertebrate and molt on days 7 to 8 (third molt) to the fourth stage and again on days 20 to 22 (fourth molt) to the adult stage.

Cultures were prepared and medium changes were made under aseptic conditions in a laminar flow hood. They were initiated with 12 to 17 third-stage larvae obtained from infected ticks (*6*). The larvae were inoculated into 16 by 125 mm glass culture tubes containing 2 ml of a 1:1 (by volume) mixture of NCTC 135 (*7*) and Iscove's modified Dulbecco's medium (IMDM) (*8*) supplemented with 5, 10, 20, or 30 percent nonheat-inactivated fetal bovine serum (FBS-NHI; M.A. Bioproducts) (*9*). The medium in the culture tubes was gassed with 95 percent N_2 and 5 percent CO_2 for 60 seconds at a flow rate of 65 ml/sec, before and after inoculation with larvae. Culture tubes were closed with nontoxic rubber stoppers and kept stationary and vertical at 36.5° ± 0.5°C. The medium was changed every 4 days (*10*).

The molt to the fourth larval stage was usually first observed between days 11 and 15 after initiation of culture. However, molting also occurred as early as day 8 and as late as day 20. Molting from the fourth stage to the adult occurred after 42 days of culture. Several hundred male and female fourth-stage larvae were obtained over the course of these studies, and a small number of them developed to adults. The growth and development of worms was influenced by the lot and concentration of the FBS supplement. Of the two lots of FBS-NHI tested, lot 948 resulted in the best development of larvae to the young adult. In media supplemented with 10 and 20 percent FBS-NHI from this lot, 50 percent (12 worms) and 40 percent (10 worms), respectively, of the worms were young adults at the termination of cultures on day 71. Only 4 percent of the worms were young adults in media supplemented with 5 percent FBS-NHI (lot 948). In media supplemented with 10 and 20 percent FBS of lot 873, 4 to 15 percent of the worms completed the fourth molt. Incomplete fourth molts, with worms tangled in partially shed cuticles or debris on the bottom of culture tubes, were seen in 4 to 25 percent of the worms in cultures supplemented with 10 or 20 percent of both lots of FBS. Most of the other worms in cultures were fourth-stage larvae, except in media supplemented with 5 percent FBS-NHI in which most worms had not developed beyond the advanced third larval stage. Survival of larvae in media supplemented with 30 percent FBS-NHI was decreased in comparison with media supplemented with 10 and 20 percent FBS. Larvae survived for 47 to 49 days,

with 57 to 82 percent having completed the third molt, but with none developing beyond the fourth larval stage. Most of the larvae from all cultures at termination were active and morphologically normal, and were at a mid to late fourth stage with well-developed reproductive systems. Small amounts of flocculent debris from the serum gradually accumulated on the bottom of culture tubes during the culture period. This amorphous material seemed to aid worms through the molting process by providing a physical substrate to which shed cuticles adhered. Other mammalian cell culture media supplemented with a number of lots and types of heat-inactivated and nonheat-inactivated sera were evaluated (*11*). Some of these supported growth, development, and molting, but none to the same degree as the medium reported here.

Worms that completed the fourth molt were active and in excellent morphological condition when the cultures were terminated on days 65 to 71. All but one of the adult worms from culture were males although we identified one additional female that had begun the fourth molt. Some other worms showing incomplete fourth molts were damaged and their sex could not be determined. The remaining female worms were fourth-stage larvae. Young adult males ranged in length from 6 to 15 mm (mean, 12 mm). The width of the body at the levels of the nerve ring and esophageal-intestinal junction ranged from 75 to 114 μm (mean, 91 μm). The lumen of the distal end of the testis was filled with many small cells, probably spermatogonia or spermatocytes, containing nuclei with distinct nucleoli. Posterior to this was a region with large, irregularly shaped cells containing large nuclei with distinct nucleoli. The larger cells, attached to the gonadal wall, were about 12 to 13 μm in diameter and were presumably secretory in nature. Further posterior was another region with many small, unidentified cells. Throughout its length, the testis did not coil upon itself. The pair of spicules did not usually protrude outside the cloaca (Fig. 1A). The adult female was 12.3 mm long and 87.5 μm wide at the level of the nerve ring and 98.8 μm wide at the esophageal-intestinal junction. The vulval opening was 630 μm from the anterior end (Fig. 1B). Posterior to the esophageal-intestinal junction the genital tube bifurcated giving rise to two branches of the genital system which extended posteriorly and terminated in large cap cells. Large, ovoid cells were present in the distal region of the longer branch of the genital tube. These larger

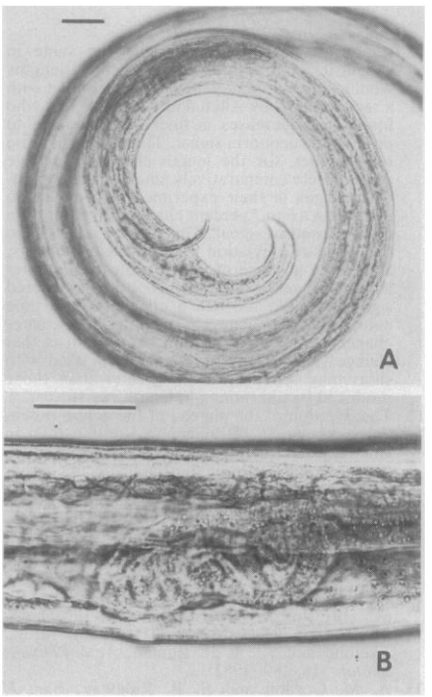

Fig. 1. Selected structures of (A) a young adult male and (B) a young adult female worm after 71 days of culture. Scale bars, 50 μm. (A) Coiled tail region with spicules protruding. (B) Lateral view of vulva and vulval opening (slightly out of focus).

cells probably represented immature oocytes. In all young adult worms, the lumen joining the esophagus and intestine was bordered by several large valve cells. The width of the intestinal lumen varied, but was usually narrower in the posterior region.

Although the growth and development of worms grown in vitro lagged behind that of worms from jirds, morphological changes occurring during development were comparable (*11*). Most aspects of the internal morphology of worms from culture were normal when compared to worms growing in vivo. Young adult worms grown in vitro were smaller in size than young adults removed from jirds at 23 days after infection. Young adult males and females from jirds were approximately 20 and 23 mm in length, respectively, whereas culture-derived males and females averaged 12 mm in length.

The high yields of well-developed fourth-stage larvae and the small numbers of young adults obtained in these cultures provide the foundation for a variety of further investigations. The growth and development of other filariids, such as the human parasites *Onchocerca volvulus*, *Wuchereria bancrofti*, and *Brugia malayi*, and the animal parasites *Litomosoides carinii* and *B. pahangi*, could also be examined in this culture sytem. The system can readily be manipulated to provide comparisons of the overall as well as specific nutritional and physicochemical requirements of the different species of filariids. The culture media and the worms developing in culture have potential application in immunological studies as sources of antigen for the diagnosis of filariasis and for possible vaccine production and testing. This culture system could also provide an in vitro test for evaluating the antifilarial activity of new compounds in a medium that supports growth, development, and molting of third-stage larvae, taken directly from the arthropod, to the fourth stage and beyond (*12*).

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10. The medium was changed by removing most of the old medium with a Pasteur pipette, leaving the worms in approximately 0.1 to 0.2 ml; 2 ml of fresh medium were then added. Tubes were again gassed with 95 percent N_2 and 5 percent CO_2. When most of the larvae had completed

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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)

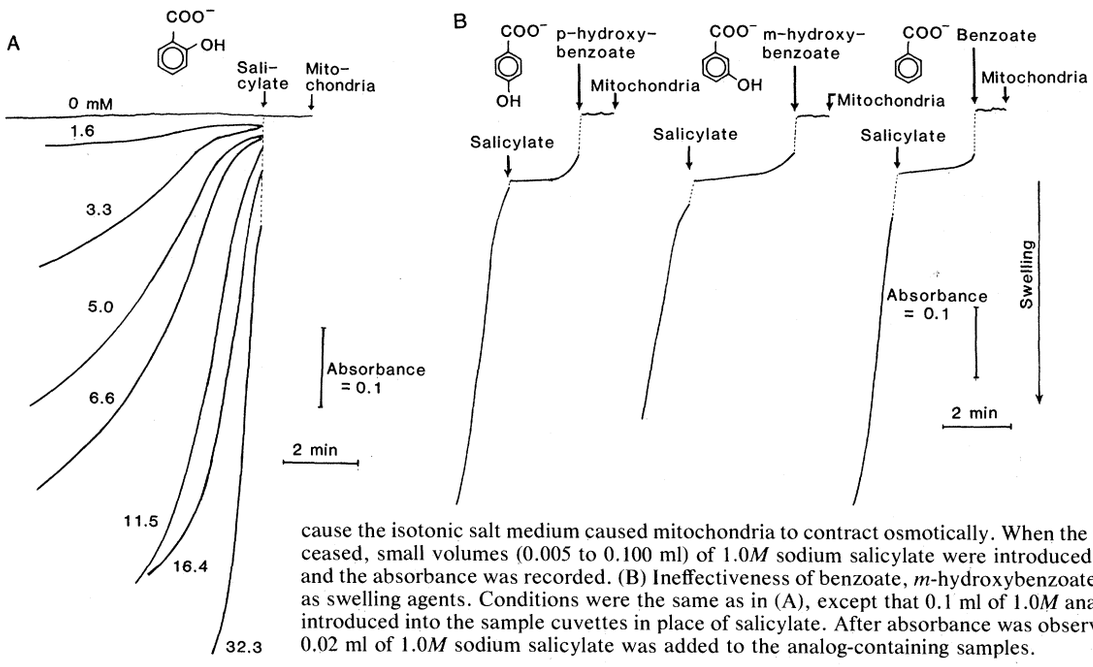

Fig. 1. Effect of salicylate and its analogs on the swelling of rat liver mitochondria. (A) Swelling as a function of salicylate concentration. The sample cuvettes contained mitochondria (1.9 mg of protein per milliliter) suspended in 3.1 ml of 20 m*M* tris and 130 m*M* NH_4Cl (*p*H 7.4). The reference 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.