Alpha Tocopherol Control of Sexuality and Polymorphism in the Rotifer Asplanchna

Abstract. *A dietary component that initiates both the transition from parthenogenetic to sexual reproduction and the production of lateral, posterodorsal, and posterior outgrowths of the body wall of females was isolated from the neutral lipids of dried grass and identified as α-tocopherol. The smallest concentrations eliciting both responses were below 100 nanograms per milliliter. Relative activities of α-, β-, and γ-tocopherol and α-tocopheryl-quinone were roughly 100 : 100 : 20 : 0.4. The synthetic antioxidants tested were without activity.*

Sexuality in *Asplanchna*, as in other rotifers in the order Ploima, results from the parthenogenetic production of mictic rather than amictic females. Both types of females are diploid and apparently identical in size and structure, but the kinds of eggs they produce differ. Amictic females produce eggs which form one polar body during maturation, are diploid, and always develop parthenogenetically into females. Mictic females produce eggs which form two polar bodies during maturation, are haploid, and develop parthenogenetically into males if unfertilized and into diploid, thick-walled resting eggs if fertilized. These sexual eggs may resume development after diapause periods of various lengths; they always hatch into amictic females.

Several clones of *A. brightwelli* Gosse produce mictic female offspring in the laboratory only when algal or higher plant material is included in the diet (*1*). The activity of this material is dependent not on chlorophyll or carotenoid pigments but on some thermostable and probably alkali-labile neutral lipid (*2*). Thus, the presence of this lipid in the diet somehow induces amictic females to produce some offspring whose eggs will undergo two maturation divisions instead of one and so become haploid.

In the closely related form *A. sieboldi* Leydig (*3*, *4*), this neutral lipid has another, very discrete effect which is correlated with the presence of algae in laboratory cultures (*3*, *5*). When amictic females ingest the lipid, their offspring are considerably larger and have four outgrowths of the body wall —two lateral, one posterodorsal, and one posterior—that are otherwise lacking. All but the posterior outgrowth are retractile and become pronounced only when extended by positive pressure inside the body cavity upon withdrawal of the head (Fig. 1). A similar but very much less extreme type of polymorphism may be induced in *A. brightwelli* (*3*, *6*).

We now report the identification of a plant neutral lipid which induces both mictic female (MF) production in *A. brightwelli* and body-wall outgrowths —commonly called humps—as well as size increase, in *A. sieboldi* (*7*).

A clone of each species was fed *Paramecium aurelia* and maintained as described (*1*). A method of preparing lipids for biological assay (*2*) has been improved (*3*). The lipid, in a small volume of acetone or ethanol, is dialyzed twice against 1000 volumes of distilled water and then against 1000 volumes of rotifer medium. The resultant emulsion is suitable for incorporation into paramecia and thus for assay. Very nonpolar lipids, such as β-carotene and hydrocarbons, emerge from dialysis as relatively large fragments and can be emulsified only by sonic disruption. These procedures were used to prepare those lipids isolated from extracts. Standard emulsions of authentic compounds were made by dissolving known amounts in traces of ethanol and pipetting the solutions into known volumes of rotifer medium.

We assayed all lipids for MF-inducing activity using *A. brightwelli* (*1*). Three animals were used for each of the several dilutions of a given lipid, and two types of control cultures were run for each assay to insure that the rotifers were sensitive to lipids of known activity and produced no mictic offspring in the absence of any added lipid. Some lipids isolated from extracts and all authentic compounds were assayed for hump-inducing activity in *A. sieboldi*. These assays were performed as the others, except that the offspring of the animals grown in the emulsions were not isolated. Each type of assay was generally repeated at least once. Hump production increases up to a point in the successive offspring of animals exposed to active lipids. Approximate relative sizes of humps in the later, maximally humped offspring were noted by observing living animals at × 75 magnifications with a stereomicroscope. Results were clear-cut and were not analyzed statistically.

Scottish Ground Grass Meal "ex Perth" (*8*) was used as a source of neutral lipid. For the first fractionation, a modification of one described earlier (*9*), 33 g of grass was extracted in a Waring Blendor with two 170-ml portions of 33 percent chloroform in methanol. The yield of lipid (1.5 g) was applied in 10 ml of redistilled *n*-hexane to a column containing a mixture of 40 g of silicic acid (Mallinckrodt), activated at 110°C, and 20 g of Hyflo Supercel. Two fractions, one eluted with hexane (450 ml) and the other with 15 percent benzene in hexane (250 ml), were collected in bulk. A fraction in 5 percent diethyl ether in hexane (400 ml), suspected of possessing activity (*3*), was collected in 8-ml subfractions. Finally, a fraction in 15 percent diethyl ether in hexane (300 ml) and one in 10 percent chloroform in methanol (300 ml) were collected in bulk. Only the second and third fractions (0.5 ml from each 8-ml sub-

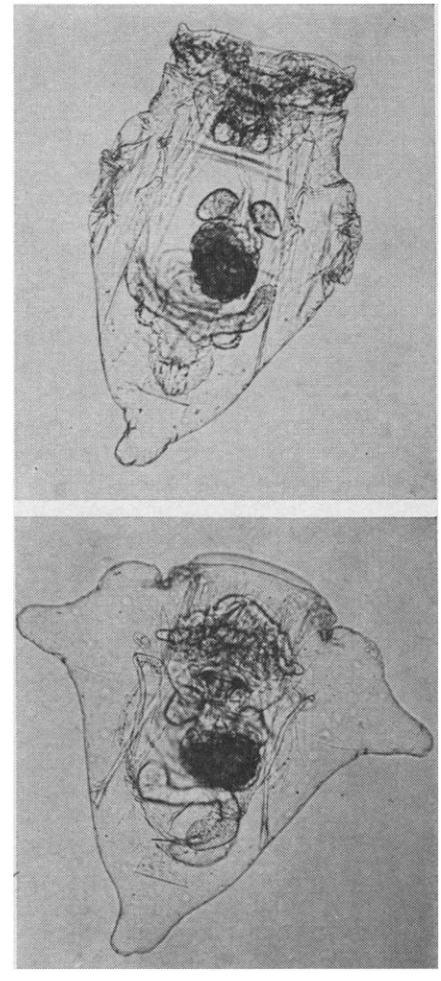

Fig. 1. Dorsoventral aspect of newly born *A. sieboldi*, about 700 μm in length, with body-wall outgrowths or humps. (Top) Animal in normal, swimming posture with lateral humps retracted; (bottom) same animal with head withdrawn and lateral humps extended. Posterior hump is plainly evident; posterodorsal hump is not visible.

fraction of the latter were pooled) possessed biological activity.

The subfractions of the third fraction were analyzed both for MF-inducing activity and for component lipids by thin-layer chromatography on silica gel G. Two solvent systems, benzene and solvent 1—a mixture of petroleum ether, diethyl ether, and acetic acid (70:30:1, by volume)—were used with appropriate neutral lipid standards. Subfractions collected before and during the early stages of triglyceride and fatty acid elution had biological activity. This result concurs with the finding (*3*) that free fatty acids from fractions in 5 percent diethyl ether in hexane were without activity and that enzymic digestion of such fractions with 0.15 percent pancreatic lipase for 2 hours at 37°C did not diminish their activity (*10*).

The activity in these subfractions was not destroyed by refluxing for 2 hours in 2*N* methanolic H_2SO_4 or by catalytic hydrogenation. One of the chromatographically purest and most active subfractions had an ultraviolet absorption maximum and minimum at 290 to 292 nm and 255 nm, respectively. The maximum did not shift after the addition of $NaBH_4$; this fact indicates the absence of a quinone. Upon thin-layer chromatography on silica gel G with benzene as solvent and iodine vapor or H_2SO_4 charring to make the organic compounds visible, this subfraction separated into two spots with R_F values of .28 and .46. Each of these areas was scraped off an untreated portion of the plate, eluted with diethyl ether, and prepared for assay in the standard manner. Only the lower spot was active. All of these data suggest that α-tocopherol might be responsible for the activity.

A fraction especially rich in the active spot was eluted from another column with increasing proportions of benzene in hexane. This fraction was further purified by removal of a wax-like material crystallizing at −20°C. Except for absorption at 5.75 to 5.80 μm, probably arising from residual wax esters, the infrared spectrum of the filtrate was very similar to that of authentic *dl*-α-tocopherol (Calbiochem).

The major component of the active filtrate migrated on silica gel G thin-layer plates with authentic α-tocopherol in three different solvent systems—benzene, solvent 1, and chloroform. Both the filtrate and authentic α-tocopherol were then treated in three different ways which convert α-tocopherol to derivatives with dissimilar R_F values—α-tocopherylquinone (*11*), α-tocopherylacetate (*12*), and α-tocopherylsuccinate (*13*). In each case, in thin-layer chromatography in solvent 1 the major component in the filtrate migrated with the authentic derivatives. In the latter two cases, scraped and eluted spots from chromatograms of control and treated filtrates demonstrated a displacement of activity consistent with the conversion of the active compound to the α-tocopherol derivative.

Table 1. Biological activities of vitamin E compounds with respect to the induction of mictic females (MF) in *A. brightwelli* and humps in *A. sieboldi*. Relative biological activity values from *A. sieboldi*.

Compound	Lowest effective concentration (ng/ml)		Relative biological activity
	MF	Humps	
dl-α-Tocopherol	14–70	14–30	100
dl-β-Tocopherol	14–70	14–30	100
dl-γ-Tocopherol	140–7000	70–140	20
α-Tocopheryl-quinone	~ 23,000	1200–5700	0.4

Direct proof for the presence and activity of α-tocopherol was obtained after the following purification steps. Lipid extracted from dried grass by hot acetone was saponified under ethanol vapor (*14*). The fraction that could not be saponified was applied to a column of silicic acid and Hyflo Supercel (2 : 1, by weight). After the β-carotene was eluted with hexane, a fraction in 2½ percent diethyl ether in hexane was collected, allowed to stand at 5°C for 24 hours, filtered through glass-fiber paper to remove crystals, and hydrogenated for 30 minutes with PtO_2 used as catalyst. This material was applied in a streak across a 1-mm thick layer of silica gel G and chromatographed in solvent 1. The silica gel in the α-tocopherol region (*15*) was scraped off, and the lipid was eluted with diethyl ether. After we repeated this step, the lipid was chromatographically pure in two solvents, benzene and solvent 1, and had an ultraviolet spectrum identical to that of authentic α-tocopherol. After exposure to iodine vapor on silica gel G (*11*), the lipid had an ultraviolet spectrum identical to that of α-tocopherylquinone. After the addition of $NaBH_4$, the spectrum was shifted to that of α-tocopherylhydroquinone. Both the purified material from grass and the authentic α-tocopherol induced the production of mictic females and humps.

Emulsions of α-tocopherol and other authentic compounds were assayed in different concentrations for MF- and hump-inducing activities in *A. brightwelli* and *A. sieboldi*, respectively. The sensitivity of *A. brightwelli* to α-tocopherol and other closely related compounds sometimes varied considerably, decreasing markedly when the general vigor and reproductive rate of the culture were low. The results with *A. sieboldi*, which always responded in a very predictable manner, were therefore somewhat more consistent. Data for the biologically active compounds tested are presented in Table 1. Vitamin K_1 (Calbiochem) and coenzyme Q_{10} (Sigma) were without activity.

Apparently the induction of humps in *A. sieboldi* is more sensitive to vitamin E compounds than is the induction of mictic females in *A. brightwelli*. However, this difference in sensitivity might simply reflect different rates of feeding and hence vitamin E concentration in the two organisms.

The relative biological activities determined for α-tocopherol, γ-tocopherol (Pierce Chemical), and α-tocopherylquinone (Nutritional Biochemicals Corp.) agree with some values obtained from other bioassays (*16, 17*). However, the similarity of the biological activities of α-tocopherol and β-tocopherol (Pierce Chemical) in the rotifer assays seems to be unique, for in other assays the relative biological activities are around 100 : 25 to 100 : 40, respectively (*16*).

There is evidence that, in some instances, α-tocopherol acts as an antioxidant and that some of the many symptoms of vitamin E deficiency reported in various vertebrates may be cured by administering synthetic antioxidants such as *N,N'*-diphenyl-*p*-phenylene-diamine (*18*). This compound (Eastman Organic Chemicals) was assayed on five occasions in seven concentrations ranging from 0.15 to 77 μg/ml. It never induced MF or hump production. Another antioxidant, butylated hydroxytoluene, also lacked activity. Therefore, α-tocopherol probably acts not as an antioxidant but by some more specific mechanism.

The control of MF and hump production by α-tocopherol and related compounds accounts for the activity of herbivorous prey organisms as well as algal and higher plant material (*3, 5*).

These compounds probably would be stored for an appreciable length of time in small algivorous rotifers, which are commonly eaten by *Asplanchna*. The absence of MF-inducing activity in the blue-green alga *Anacystis nidulans* (*2*) is now also explicable, for this organism is devoid of α-tocopherol and α-tocopherylquinone (*19*).

The function of vitamin E compounds in invertebrates is very poorly known. They have been suspected of enhancing vigor and fecundity in the cladoceran *Daphnia* (*20*); they stimulate egg sac formation in copepods (*21*) and are essential for spermatogenic activity in the cricket *Acheta* (*22*). Our report appears to be the first to show that these compounds can initiate either a transition from parthenogenetic to sexual reproduction or a marked change in body morphology.

The vitamin E control of MF production in *Asplanchna* could be very adaptive if this vitamin was required for spermatogenesis, as it is in crickets, for it would benefit a population to make mictic females only under conditions when functional males could be produced. Hence a possible explanation for the biological significance of the response to plant lipids considered earlier (*2*) can now be offered.

There are many occasions when the biological activities of natural and synthetic vitamin E compounds need to be determined. For these, bioassays using the MF- and hump-producing responses of *A. brightwelli* and *A. sieboldi* appear admirably suited. Although the responses are probably very complex, they are discrete, extremely sensitive, and very easily and quickly detected. Furthermore, the practical convenience of this system over those requiring tedious maintenance of vertebrates deficient in vitamin E is obvious. Also for these reasons, *Asplanchna* may prove useful in studies of the mechanism of the action of vitamin E.

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15. Authentic α-tocopherol in marker lanes was made visible with an iodine spray, while the rest of the plate was protected by glass.
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Myoglobin Subfractions: Abnormality in Duchenne Type of Progressive Muscular Dystrophy

Abstract. *Human metmyoglobin was separated electrophoretically into four subfractions: Mb_1, Mb_2, Mb_3 and Mb_4, which divide into at least two biochemically independent groups: Mb_1 and Mb_2, and Mb_3 and Mb_4. In normal subjects, Mb_1 constituted the predominant component; Mb_2, Mb_3, and Mb_4 were the minor components in this descending order. In the Duchenne type of progressive muscular dystrophy, on the contrary, a remarkable decrease in Mb_1 and a concomitant increase in Mb_3 were observed. This unique abnormality in the relative distribution of myoglobin subfractions was recognized only in the Duchenne type and not in other types of progressive muscular dystrophy or in other myopathies.*

We have already reported (*1*, *2*) that the absorption maximum in the ultraviolet spectrum of metmyoglobin, in the Duchenne type of progressive muscular dystrophy (PMD), was about 275 mμ, in contrast to the value of 281 mμ for metmyoglobin from the other types of PMD, spinal progressive muscular atrophy, polymyositis, myoglobinuria, and normal subjects. Further studies of the myoglobin of PMD have shown that this spectral abnormality is ascribable to an abnormal distribution pattern of myoglobin subfractions.

Rossi-Fanelli *et al.* (*3*) and Perkoff *et al.* (*4*) reported that human skeletal myoglobin was heterogeneous and was separated into three to four components by paper electrophoresis or DEAE cellulose–column chromatography. Kossman *et al.* (*5*), however, reported that minor components of myoglobin were artifactual. Existence of the myoglobin subcomponents, therefore, is still problematical, and rigorous physicochemical characterization of the subcomponents has not been reported.

Perkoff (*6*) claimed that myoglobin was separated into three fractions on DEAE cellulose–column chromatography, and named them F_1, F_2, and F_3. He observed that several diseases, including childhood dystrophy, dermatomyositis and myoglobinuria, caused a decrease in F_1 and F_2, with a concomitant increase in F_3; there was similar change in fetus myoglobin.

In our studies the metmyoglobin was isolated and purified by Singer's method (*1*) from the skeletal muscles of normal subjects and patients having various myopathies. The metmyoglobin