Sterol Biosynthesis in the Free-Living Nematode *Panagrellus redivivus*

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The fate of the known sterol precursor squalene 2,3-oxide was investigated in the free-living nematode *Panagrellus redivivus*. The nematodes were cultured axenically in the presence of [4-³H]squalene 2,3-oxide. Radioactivity was found in the total lipids of the isolated nematodes. Essentially all of the radioactivity encountered in the total lipids was found in the non-saponifiable fraction. The components present in the non-saponifiable fraction were separated and isolated by t.l.c. Three labelled components were identified by a combination of t.l.c., g.l.c. and mass spectroscopy. It is established that *P. redivivus* has the capacity for biosynthesis of lanosterol. No labelled C₂₇ sterols could be detected.

The biosynthetic pathway for synthesis of cholesterol from acetate has been studied extensively, and, at one time, it was believed that the capacity for sterol biosynthesis was universal among eukaryotic organisms (Haslewood, 1960). The capacity for sterol biosynthesis is known to be completely lacking only in prokaryotic organisms (Asselineau & Lederer, 1960; Bloch, 1965). Higher plants and animals seem able to effect complete sterol biosynthesis *de novo* from acetate (Nes, 1971). Among the invertebrate phyla, however, there are groups of organisms that appear to have lost the ability to synthesize sterols from short-chain precursors. Insects characteristically require dietary sources of cholesterol or phytosterols for larval growth and development (Lehoux & Sander, 1970). Cholesterol functions in cell-membrane structure and serves as a precursor of hormones regulating moulting and metamorphosis of insects (Clayton, 1964). The dependence of insects on exogenous sterols throughout their life cycles is a major biochemical difference from vertebrates and indicates that insects lack the capacity for complete sterol biosynthesis *de novo* (Robbins *et al.*, 1971).

There is strong evidence that some members of the phylum Aschelminthes (nematodes) have lost the capacity for sterol biosynthesis *de novo*. Nematodes have been shown to require exogenous sterols for axenic culture (Dutky *et al.*, 1967; Hieb & Rothstein, 1968; Cole & Dutky, 1969; Hieb *et al.*, 1970) and are unable to incorporate labelled acetate or mevalonate into sterols (Cole & Krusberg, 1968; Rothstein, 1968). However, it is reported that squalene and lanosterol support minimal development in larvae of the animal-parasitic nematode *Nippostrongylus brasiliensis* (Bolla *et al.*, 1972). The sterol precursor lanosterol has been identified as a normal constituent of the lipids of the free-living nematode *Panagrellus redivivus* (Willett & Downey, 1973).

The present paper reports the results of our studies on the fate of the known sterol precursor squalene 2,3-oxide in the free-living nematode *P. redivivus*, which requires a dietary source of sterols (Hieb *et al.*, 1970). It has also been shown to be incapable of synthesizing sterols from either acetate or mevalonate (Rothstein, 1968). We have found that *P. redivivus* can convert the sterol precursor squalene 2,3-oxide into lanosterol. It appears therefore that these organisms retain the cyclizing enzyme, suggesting that the block in the pathway for sterol biosynthesis occurs between lanosterol and cholesterol.

Experimental

Methods

Culture of nematodes. Nematodes (*P. redivivus*) were cultured axenically at 20–23°C in a medium containing 4.0g of soya peptone, 3.0g of yeast extract and 10ml of heated liver extract in 100ml of distilled water. The heated liver extract was prepared by the method of Cryan *et al.* (1963). The soya peptone and yeast extract were purchased from General Biochemicals Inc., Laboratory Park, Chagrin Falls, Ohio 44022, U.S.A. After a 7-day growth period, the nematode population averaged 200 individuals/ml of growth medium. The nematodes were harvested at the end of a 7-day growth period and then maintained for 24h in a 2% (w/v) trypsin (Digestive Ferments Co., Detroit, Mich., U.S.A.)–phosphate solution to solubilize precipitated protein present in the medium. The trypsin–phosphate solution was prepared by adding 1 vol. of 1M-phosphate buffer (pH 8) to 3 vol. of 2% (w/v) trypsin in sterile distilled water. Trypsin–phosphate solution (2ml) was added for every 5ml of medium present in the harvest. The nematodes were separated by centrifugation

(10 min, 1000 g_{av.}) and then washed twice with water, frozen (with solid CO₂-acetone mixture), and freeze-dried [24 h in a Virtis Lyophilizer; Viritis Company Inc., Gardiner, New York, N.Y. 12525, U.S.A. at -50°C and 3.33-4.44 Pa (0.025-0.03 Torr)].

For the experiment, nematodes were cultured as described above in the presence of 1200 µg of [4-³H]-squalene 2,3-oxide [specific radioactivity 17050 d.p.m./µg; prepared by the method of Nadeau & Hanzlick (1969)], introduced into 40 ml of medium as a sterile solution in Tween 80 (Nutritional Biochemicals Corp., Cleveland, Ohio 44128, U.S.A.). Upon harvest, 130.6 mg of freeze-dried nematodes was obtained. Two controls on the experiment were used. Control 1 contained medium and [4-³H]squalene 2,3-oxide but no nematodes; control 2 contained medium and nematodes but no labelled squalene 2,3-oxide.

Total lipid and non-saponifiable material. The total lipid was extracted from the freeze-dried nematodes by the method of Folch *et al.* (1957). The extracted lipid (20.0 mg) represents 15.4% of the dry weight of the nematodes. The extracted lipid was then saponified by treatment with a 15% (w/v) solution of KOH in ethanol (14 h at room temperature) (Clayton *et al.*, 1963). The non-saponifiable material (2.7 mg) was isolated and subjected to t.l.c. for separation into its components. Isolation of the total lipids and the non-saponifiable material from the controls was done in the same manner.

T.l.c. This was carried out on 5 cm × 20 cm glass plates coated with 0.25 mm-thick layers of methanol-washed silica gel G [10% (w/w) CaSO₄ binder] (E. Merck, Darmstadt, Germany), which were dried for 30 min at 120°C before use. Three solvent systems were used for separating the non-saponifiable material. All systems consisted of ethyl acetate-hexane mixtures. System A was 25% (v/v) ethyl acetate-hexane; system B, 75% (v/v) ethyl acetate-hexane; and system C, 5% (v/v) ethyl acetate-hexane. Components were localized on the plates by exposure to I₂ vapour for a few seconds. Radioactive incubation products were localized by scanning the plates for radioactivity with a Packard Model 7201 radio-chromatogram scanner (Packard Instrument Co. Inc., Downers Grove, Ill. 60515, U.S.A.). The separated bands were then scraped from the plates and extracted with methylene chloride-methanol (1:1, v/v). This solvent mixture was used for extracting materials contained in all bands except those thought to contain squalene 2,3-oxide, for which methylene chloride alone was used (to avoid the possibility of hydrolytic attack on the epoxide ring). Extractions were carried out by scraping the silica gel into a disposable pipette plugged with glass wool and rinsing the material from the silica gel with 4-6 vol. of the methanol-methylene chloride mixture. Radioactivity measurements were made in a Packard

liquid-scintillation counter (Model 2002) operating with an efficiency of 57.8% for ³H. Samples were assayed in solution in a scintillation fluid containing 5 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre of toluene. These materials were all purchased from Packard Instrument Co.

G.l.c. This was carried out on a Hewlett-Packard Model 402 gas-liquid chromatograph with all-glass columns (1824 mm × 3 mm inside diameter) and equipped with a flame ionization detector and an effluent stream splitter. Materials corresponding to emergent peaks were collected by inserting glass capillary tubes of approx. 1 mm bore through a Teflon gasket attached to the outlet of the gas chromatograph. With the splitter valve open all the way the efficiency of collection by this technique was 95-98% of the injected sample. Varian STAP (steroid analysis phase) was used as the liquid phase on Chromosorb G, 100 mesh (both purchased from Varian Aerograph, Walnut Creek, Calif., U.S.A.), at 5% (w/w) concentration. The columns were preconditioned at 250°C for 48 h and then operated as a routine at 230°C with inlet and outlet temperatures of 250°C and a helium flow rate of 80 ml/min.

Trimethylsilyl ethers were prepared by treating a sample of the sterol material (less than 1.0 mg) with 100-200 µl of trimethylchlorosilane (Pierce Chemical Co., P.O. Box 117, Rockford, Ill. 61105, U.S.A.) and 0.5 ml of anhydrous pyridine for 30 min at room temperature. The mixture was then evaporated to dryness under a stream of N₂ and the material redissolved in hexane plus a drop or two of water (to dissolve pyridine salts). This hexane-water mixture was filtered through anhydrous Na₂SO₄, evaporated to dryness, and the residue dissolved in dry hexane for injection into the gas chromatograph. 5α-Cholestane (Mann Research Laboratories, New York, N.Y. 10006, U.S.A.) was used in the injected samples as a reference compound. Retention times are quoted in relation to that of cholestane as R_C values.

Mass spectroscopy. Mass spectra were obtained with the use of a Hitachi RMU-6E single-focussing mass spectrometer at 70 eV.

Materials

Lanosterol. Lanosterol (Mann Research Laboratories) was recrystallized twice from ethanol, giving material with m.p. 140°C showing one spot on t.l.c., R_F 0.46 (system A). The trimethylsilyl ether of this twice-recrystallized material gave one peak on g.l.c., R_C 3.02.

Squalene 2,3-oxide. Squalene 2,3-oxide was prepared from all-*trans*-squalene by the method of van Tamelen & Curphey (1962) [all-*trans*-squalene was obtained by purification of the commercial

material (Nutritional Biochemicals Corp., Cleveland, Ohio 44128, U.S.A.) by chromatography on silicic acid followed by thiourea clathrate formation (Nicolaidis & Laves, 1954)]. The oxide has R_F 0.86 in system A.

Squalene 2,3-glycol. Squalene 2,3-oxide (0.5g) was dissolved in 1ml of tetrahydrofuran; water was added until the solution became cloudy, then additional tetrahydrofuran until the solution became clear. To this was added 0.42ml of 3% (w/v) HClO_4 and enough tetrahydrofuran to obtain a clear solution. The solution was left at 2°C for 18h under N_2 . The tetrahydrofuran was then evaporated under N_2 and the product extracted four times with portions (1ml) of *n*-hexane. The combined extracts, dried over anhydrous Na_2SO_4 and concentrated in a stream of N_2 , gave 0.55g of squalene 2,3-glycol. A sample of this material subjected to t.l.c. in system A gave one spot at R_F 0.26 and a single spot R_F 0.78 in system B.

1,1',2-Trisnorsqualene-3-aldehyde. Squalene 2,3-glycol (37mg) was dissolved in 1ml of tetrahydrofuran and 250mg of sodium metaperiodate was added. Sufficient water (1.5ml) to dissolve the sodium metaperiodate was added. The resulting solution was mixed well, saturated with N_2 , and left at 2°C for 24h. The tetrahydrofuran was removed in a stream of N_2 and the product extracted five times with portions (1ml) of *n*-hexane. The extracts, combined, dried and evaporated under N_2 , gave 26.3mg of 1,1',2-trisnorsqualene-3-aldehyde.

T.l.c. of this material gave single spots in both systems A and C, with R_F values of 0.86 and 0.54, respectively. G.l.c. analysis showed a single peak with $R_c = 1.49$.

Results

Incorporation of squalene 2,3-oxide

The nematodes were supplied with 1200 μg of $[4\text{-}^3\text{H}]\text{squalene 2,3-oxide}$ having a specific radioactivity of 17050 d.p.m./ μg . Of the total activity supplied to the nematodes (20.46×10^6 d.p.m.), 98.1% (20086080 d.p.m.) was recovered with the 'spent medium' (culture material minus the nematodes), and, on t.l.c. analysis, proved to be recovered squalene 2,3-oxide. This represents an incorporation into the nematodes of 1.9% of the total radioactivity supplied.

T.l.c. of non-saponifiable material

A Folch extraction (Folch *et al.*, 1957) on the freeze-dried nematodes (130.6mg dry wt.) gave 20.0mg of total lipids (15.4% of the dry weight of the nematodes), with a radioactivity of 356000 d.p.m. Thus 90% of the radioactivity taken up by the nematodes is found in the extracted total lipids. Saponification of the total lipid fraction gave 2.7mg (2.0%,

w/w) of non-saponifiable material with a total radioactivity of 348570 d.p.m. This represents 98% recovery of the radioactivity associated with the total lipid.

The non-saponifiable material, after t.l.c. on silica gel in system A, gave nine distinct bands (experimental, bands I-IX). Three of these bands showed radioactivity when the plate was scanned; the other six bands were not radioactive. The radioactivity was associated with bands (IV), (V) and (VIII). Bands (IV) (R_F 0.27), (V) (R_F 0.46), and (VIII) (R_F 0.87) contained 84580, 3760 and 206490 d.p.m. respectively. The total radioactivity recovered from these three bands (294830 d.p.m.) represented 84.5% of the radioactivity associated with the non-saponifiable material before chromatography.

Control 1, which contained no nematodes, showed six of the same non-radioactive bands as the experimental material, in addition to one radioactive band arising from labelled squalene oxide. This seems to indicate that the six non-radioactive bands were not associated with the nematodes but probably arose from the medium. The $[4\text{-}^3\text{H}]\text{squalene 2,3-oxide}$ band in control 1 (band VI) had the same R_F value as band (VIII) of the experimental material. Control 2 also showed the same six bands plus one (band IV) with the same R_F value as band (V) of the experimental material. These results are summarized in Fig. 1.

Identification of band (VIII) (experimental) as unchanged squalene 2,3-oxide

Since control 1, containing $[4\text{-}^3\text{H}]\text{squalene 2,3-oxide}$ but no nematodes, showed one active band with the same R_F value as band (VIII) of the experimental material, and control 2, containing nematodes but no squalene oxide, showed no band at this R_F value, and since authentic squalene 2,3-oxide had the same R_F value as band (VIII), it seemed reasonable to conclude that band (VIII) could be unchanged squalene 2,3-oxide.

Conversion into squalene 2,3-glycol. Material from band (VIII) (experimental) containing 88100 d.p.m. (suspected squalene 2,3-oxide) was converted into the glycol (see the Experimental section). The resulting material (76840 d.p.m., 87.2% recovery) was subjected to t.l.c. on silica gel in both systems A and B and gave single spots with R_F values of 0.26 and 0.78 respectively. These R_F values are identical with R_F values of authentic squalene 2,3-glycol in these solvent systems.

Identification of band (IV) (experimental) as squalene 2,3-glycol

It was noted that the R_F of band (IV) (experimental) in solvent system A was the same as that of squalene

Fig. 1. T.l.c. chromatography of non-saponifiable lipids from *P. redivivus*

Material was chromatographed on silica gel G with 25% (v/v) ethyl acetate in hexane. Asterisks indicate radioactive bands, numbers refer to R_F values. Experimental, growth medium + nematodes + [^3H]squalene 2,3-oxide; control 1, medium + [^3H]squalene 2,3-oxide (no nematodes); control 2, medium + nematodes (no labelled oxide).

2,3-glycol in that system (0.26). A portion of band (IV) material was then subjected to t.l.c. in solvent system B and the R_F value compared with that of authentic squalene 2,3-glycol. The identity of the R_F values suggested that band (IV) might be [^3H]squalene 2,3-glycol. As further evidence for this identity, a sample of band (IV) (48210 d.p.m.) was converted into 1,1',2-trisnorsqualene-3-aldehyde. T.l.c. of the material obtained (41340 d.p.m., 85.7% recovery) on silica gel with solvent systems A and C gave R_F values of 0.86 and 0.54 respectively and compare favourably with those of authentic 1,1',2-trisnorsqualene-3-aldehyde.

A portion of the aldehyde material was subjected to g.l.c. analysis. The material showed a single peak

with R_C 1.47, identical with that of authentic aldehyde. A sample subjected to purification by g.l.c. gave material with a specific radioactivity of 478 d.p.m./ μg . The expected specific radioactivity of this material, calculated from the radioactivity of band (IV) material, was 489 d.p.m./ μg .

In addition, the mass spectrum of the aldehyde material prepared from band (IV) was identical with that of authentic 1,1',2-trisnorsqualene-3-aldehyde.

Identification of band (V) (experimental) material as lanosterol

From the assumption that band (V) (experimental) might be a sterol, a sample was converted into the trimethylsilyl ether. This material was then subjected to g.l.c. and showed one radioactive peak at R_C 3.02, quite similar to the retention time shown by authentic lanosterol trimethylsilyl ether (R_C 3.03). The R_F of band (V) in t.l.c. system A (0.47) was also quite similar to that of lanosterol in the same solvent (0.46). The specific radioactivity of the trimethylsilyl ether of band B was 33.0 d.p.m./ μg ; the specific radioactivity of untreated material from band (V) was 37.6 d.p.m./ μg . The expected radioactivity of lanosterol trimethylsilyl ether, calculated from the specific radioactivity of unchanged band (V) material, would be 32.2 d.p.m./ μg .

As further proof of the identity of band (V), the mass spectrum of the trimethylsilyl ether of band (V) material was identical with that of authentic lanosterol trimethylsilyl ether.

Discussion

Substrate (squalene oxide) of high specific radioactivity was used in these experiments because of the expected limited incorporation *in vivo*. The nematodes were given [^3H]squalene 2,3-oxide under normal growth conditions, and radioactivity was found in the total lipids extracted from the harvested nematode population. Separation of the non-saponifiable material by t.l.c., in a solvent system that separates neutral lipids, gave three distinct radioactive bands. This result demonstrates that the labelled epoxide is taken into the nematodes under the conditions of the experiment. The control showed no labelled materials other than the starting epoxide; thus no incidental synthesis of artifacts occurs. The active bands were lanosterol, squalene 2,3-oxide, and squalene 2,3-glycol, a product of hydrolysis of the epoxide. No labelled C_{27} sterols were detected.

In previous experiments investigating incorporation of labelled mevalonic acid in *P. redivivus*, radioactivity was observed in the non-saponifiable fraction, but the exact nature of the radioactive material was not ascertained. Cole & Krusberg (1968)

observed radioactivity in the sterol fraction from *Turbatrix aceti* grown in the presence of [^{14}C]-mevalonate, which was lost after the attempted preparation of a dibromide and recrystallization. In another experiment with [^{14}C]-mevalonate and *T. aceti*, Rothstein (1968) observed a radioactive material in the sterol fraction which was not cholesterol. The identity of this material was not ascertained.

The present work with *P. redivivus* has demonstrated that radioactivity from [^3H]-squalene 2,3-oxide is incorporated into lanosterol but not into cholesterol or other sterols and demonstrates that the nematode retains one of the key enzymes in sterol biosynthesis, 2,3-oxidosqualene lanosterol cyclase. The sterol requirement of nematodes may thus be attributed to an interruption of cholesterol biogenesis after cyclization of the lanosterol stage. It is possible that the block in the pathway of sterol biosynthesis in invertebrates may result from loss of one or more of the enzymes responsible for oxidative removal of the methyl groups at C-4 and C-14 of the steroid ring system.

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