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WHAT ABOUT SQUALENE IN THE EARTHWORM *LUMBRICUS TERRESTRIS*?

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Abstract—1. It is shown that *Lumbricus terrestris* does not utilize ^{14}C-labelled squalene for the biosynthesis of sterols.

2. The squalene administered was metabolized rapidly and radioactivity was recovered in several lipids, making it unlikely that accumulation of radioactivity in squalene might occur.

3. The distribution of radioactivity in the hydrocarbons of *L. terrestris* after the administration of sodium acetate-1-^{14}C was investigated.

4. Both saturated and unsaturated straight-chain and branched hydrocarbons were radioactive.

5. Squalene was present in a very small quantity and may be of exogenous origin.

INTRODUCTION

IN THE phylum of the Annelida, *Lumbricus terrestris* seems to hold an exceptional position with reference to its capacity of synthesizing 3β-sterols. Whereas several polychaetous annelids can synthesize sterols from acetate or mevalonate (Wootton & Wright, 1962; Walton & Pennock, 1972; Voogt, 1973) and this has been shown also for the oligochaetes *Tubifex tubifex* and *Enchytraeus albidus* (Voogt, 1974), it seems well established that the earthworm *L. terrestris* does not synthesize sterols (Wootton & Wright, 1960, 1962; Voogt, 1974).

In 1960 Wootton & Wright observed that after the administration of DL-mevalonic acid-2-^{14}C to *L. terrestris* no radioactivity had been incorporated into the sterols, but was mainly recovered in the intermediate squalene. This compound was purified by preparing the squalenehexahydrochloride. In 1962 the same authors (Wootton & Wright, 1962) reported that 96 hr after the administration of DL-mevalonic acid-2-^{14}C to *Lumbricus* nearly half of the radioactivity of the non-saponifiable lipids was present in squalene. From these results, Wootton & Wright concluded that *L. terrestris* is incapable of cyclizing squalene to lanosterol and that a genuine metabolic block exists in the earthworm. Obviously these conclusions are fully dependent on the actual presence of squalene. This prompted the question whether *Lumbricus* is indeed incapable of cyclizing squalene when this compound is administered to the animal and further, whether, if cyclization is impossible, squalene is accumulated in the animal. To answer these questions two experiments were conducted in which either radioactive squalene or acetate was administered to *L. terrestris*.

MATERIALS AND METHODS

Sodium acetate-1-^{14}C (sp. act. 1 mCi/1·49 mg) was purchased from New England Nuclear Corporation. The ^{14}C-labelled squalene used was biosynthesized by rats (Rao & Olson, 1967).

Six rats, fed during 5 days on a normal diet enriched with 5% cholesterol, each received 5 μCi of DL-mevalonic acid-2-^{14}C (DBED salt, New England Nuclear Corporation) dissolved in 0·3 ml ethanol. One hr after the injection the animals were decapitated and the livers removed immediately. Lipids were extracted from the livers using the procedure of Bligh & Dyer (1959). The hydrocarbon fraction was obtained by means of column chromatography according to Carroll (1961). Gas chromatography of this fraction showed that it contained about 95% squalene. This fraction was not purified further.

Animals

A batch of *L. terrestris* (fresh weight 119·9 g) was collected in August 1969 in the neighbourhood of Utrecht. A number of the animals (total fresh weight 31·2 g) received by injection in total about 0·4 μCi of the ^{14}C-labelled squalene dissolved in 1·1 ml hexanol. The hexanol was used because it possessed better dissolving properties for squalene than ethanol whilst it was only slightly more toxic. After injection the animals were put back with the non-injected animals. Incubation lasted for 40 hr.

Another batch of *L. terrestris* (thirty-five animals, total fresh weight 88·5 g) was obtained by the generosity of Dr. J. Doeksen, Institute for Biological and Chemical Research on Field Crops and Herbage, Wageningen, The Netherlands. These animals were each injected with an aqueous solution of sodium acetate-1-^{14}C. Incubation was terminated after 48 hr.

These experiments will be indicated further by experiments A and B, respectively.

Extraction, saponification and purification of lipids

Lipids were extracted from the animals using the procedure of van der Horst *et al.* (1969). Lipids from experiment A were saponified in a solution of 1·5 N KOH in 80% methanol under the usual conditions. The isolation of saponifiable and non-saponifiable lipids and the separation of the non-saponifiable lipids into a crude squalene fraction, a crude sterol fraction and a remainder fraction were performed as described elsewhere (Voogt, 1971a, b). Sterols were isolated from the crude sterol fraction via their digitonides.

Lipids from experiment B were separated into several lipid classes, according to Carroll (1961). Sterols were purified by chromatographing them once on thin-layer plates in toluene–ethyl acetate (4 : 1, v/v) and after hydrogenation and acetylation in hexane–diethyl ether (9 : 1, v/v).

The hydrocarbon fraction of experiment B was separated into saturated and unsaturated hydrocarbons by means of column chromatography on Adsorbosil (Applied Science Laboratories) using for development hexane and hexane–diethyl ether (90 : 10, v/v), respectively. The unsaturated hydrocarbons were separated into straight chain and branched by means of thiourea (Chaykin, 1966). The branched unsaturated hydrocarbons were fractionated according to the degree of unsaturation on thin-layer plates impregnated with 25% silver nitrate using for development hexane–diethyl ether (95 : 5, v/v), modified after Streibl & Stránský (1968).

Measurement of radioactivity

Radioactivities were measured with a Packard Liquid Scintillation Spectrometer, Model 3320, using toluene containing 0·4% Omnifluor (NEN Chemicals) as the scintillation cocktail.

Gas chromatography of the hydrocarbons

Hydrocarbons were analysed on a Becker Instrument, Model 2300, with dual columns and FID. The columns (180 × 0·4 cm i.d.) were filled with either Chromosorb W (60–80 mesh, acid washed) coated with 20% PEGA (polyethyleneglycol adipate) + 3% H_3PO_4 or Chromosorb W (100–120 mesh, acid washed, inactivated with 5% DMCS) coated with 4% SE-52. The column temperature amounted to 170°C.

DISCUSSION

After the lipids had been extracted from the animals, used in the experiment with radioactive squalene (experiment A), they were fractionated and the specific radioactivities were determined. The results are given in Table 1. This table shows that both saponifiable and non-saponifiable lipids were radioactive, radioactivity in the latter fraction far exceeding that in the saponifiable lipids. It also shows that after saponification 30 per cent of the radioactivity could not be extracted from the saponification mixture by means of petroleum ether (b.p. 40–60°C). A relatively small portion of the radioactivity in the unsaponifiable lipid was recovered in the crude squalene and sterol fraction and since the remaining fraction contained 20·98 per cent of the radioactivity, about 30 per cent of the radioactivity present in the total lipids was retained on the alumina column. These data clearly show that the squalene injected was intensively metabolized by the animals. After purification the specific radioactivity of the sterols amounted to 6 dis min^{-1} per mg. This value indicates that the cyclization of squalene hardly proceeds to any extent and seems to confirm the hypothesis of Wootton & Wright (1960, 1962) that squalene cannot be cyclized by *Lumbricus*. However, an accumulation of radioactivity in squalene, after the administration of mevalonate to *Lumbricus* as was described by Wootton & Wright (1960, 1962), seems to be unlikely as the foregoing experiment shows that squalene is quickly metabolized.

Table 1. Radioactivity of the lipid fractions isolated from *L. terrestris* expressed in dis min^{-1} per mg and as a percentage of the radioactivity incorporated in the total lipids after the administration of ^{14}C-labelled squalene

Lipid fractions	dis min^{-1} per mg	% of radioactivity in total lipids
Total lipids	1132	
Saponifiable lipids	442	1·00
Unsaponifiable lipids	6398	69·28
Crude squalene fraction	4008	4·50
Crude sterol fraction	560	4·42
3β-Sterols	35	0·15
3β-Sterols after purification	6	

To check this accumulation special attention was paid to the hydrocarbon fraction of the lipids from experiment B. Incorporation of radioactivity into the total lipids amounted to 0·90 per cent of the total dosage administered, corresponding to a specific radioactivity in these lipids of 1319 dis min^{-1} per mg. After purification the sterols showed a specific radioactivity of 1·5 dis min^{-1} per mg. This means that biosynthesis of sterols had not occurred. The hydrocarbon fraction was isolated and fractionated. The results are summarized in Table 2.

This table shows that the total radioactivity in the hydrocarbons amounted to about 160,000 dis min^{-1}, being 4 per cent of the activity in the total lipids. These hydrocarbons were fractionated by means of column chromatography on Adsorbosil. Despite the great loss of material (and radioactivity) it is clear that most hydrocarbons are unsaturated. The specific radioactivity of the unsaturated hydrocarbons is lower, but their total radioactivity is higher than that of the saturated ones. The unsaturated hydrocarbons were separated into straight chain and branched by means of thiourea. Losses are considerable and unexplained. Specific radioactivities of both fractions are lower than that of the parent fraction. This indicates that a fraction with a

Table 2. Quantities and relative weights as well as the specific and total radioactivity in the hydrocarbon fraction of *L. terrestris*

Hydrocarbon fraction	mg	% of total hydrocarbons	dis min^{-1} per mg	dis min^{-1}	% of radioactivity in total hydrocarbons
Total hydrocarbons	60·16		2671	160,687	
(% of total lipids)	2·27				
(% of radioactivity in total lipids)			4·0		
Saturated hydrocarbons	17·98	29·89	3481	62,588	39·0
Unsaturated hydrocarbons	35·56	59·51	2043	72,649	45·2
Unsaturated hydrocarbons with straight chain	9·46	15·72	1930	18,258	11·4
Unsaturated hydrocarbons with branched chain	13·86	23·04	1729	23,964	14·9
Squalene fraction				40	

high specific radioactivity must have been lost. The amount of branched unsaturated hydrocarbons exceeds that of the straight ones, but their specific radioactivity is lower. Thin-layer chromatography on silver nitrate-impregnated silicagel G of the unsaturated hydrocarbons yielded several bands, corresponding with the degree of unsaturation. The zone co-chromatographing with reference squalene was eluated. Gas chromatography of this fraction showed that it contained several components, squalene being by far the largest. The weight of the total fraction was less than 0·01 mg and the total radioactivity amounted to 40 dis min^{-1}.

This experiment clearly shows that only minute quantities of squalene are present in the earthworm. Further, it is quite uncertain whether the 40 dis min^{-1} mentioned above are from the squalene, because several other components were present. How this may be, our results differ strongly from those of Wootton & Wright (1960, 1962). The results of experiment A made it unlikely that accumulation of radioactivity might occur into squalene after the administration of a sterol precursor. The results of experiment B even suggest that it is unlikely that squalene is synthesized at all and thus may originate from the diet. In that case the absence of sterol biosynthesis in *Lumbricus* is not only due to the incapacity of this animal to cyclize squalene, but also to its incapability of performing at least one other step in the biosynthesis of sterols, which is situated in this pathway prior to squalene.

All data obtained up till now agree in that *Lumbricus* is incapable of synthesizing sterols. It is likely that the animal can support its need for sterols from the diet. This may have a high content of phytosterols. The way in which *Lumbricus* utilizes these sterols to obtain its own specific sterol composition will be studied.

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