Comp. Biochem. Physiol., 1972, *Vol.* 42**B**, *pp.* 15 *to* 22. *Pergamon Press. Printed in Great Britain*

HYDROCARBONS IN THE MILLIPEDE *GRAPHIDOSTREPTUS TUMULIPORUS* (KARSCH) (MYRIAPODA: DIPLOPODA)—I. *IN VIVO* INCORPORATION OF ^{14}C-LABELLED PRECURSORS INTO THE HYDROCARBON FRACTION

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(*Received* 7 *October* 1971)

Abstract—1. Biosynthesis of hydrocarbons in male and female specimens of the millipede *Graphidostreptus tumuliporus* was investigated after injection of the following precursors: 1-^{14}C-acetate, 16-^{14}C- and 1-^{14}C-palmitic acid, isoleucine-$^{14}C(U)$, valine-$^{14}C(U)$ and mevalonic-2-^{14}C acid.

2. Both sexes are capable of synthesizing hydrocarbons from all precursors used. High radioactivities were achieved with acetate, palmitic acid and mevalonic acid, whilst with isoleucine and valine there was but little radioactivity in the total lipids and hydrocarbons.

3. Rapid incorporation of mevalonic acid into the hydrocarbons may indicate that the branched hydrocarbons possess an isoprenoid type of branching.

INTRODUCTION

THE LARGE surface to volume ratio of insects and other arthropods makes it necessary, especially for land-living species, that excessive evaporation has to be prevented. In maintaining the water balance cuticular lipids play an important role and these components also protect the animals from several external influences like penetration of micro-organisms and of some inorganic chemicals (Wigglesworth, 1957; Beament, 1964; Gilby, 1965).

In insects, hydrocarbons are the most important components in the lipids of the cuticle, although there are specialized species which form wax esters of long-chain fatty acids and alcohols, e.g. in scale insects (Tamaki, 1966; Tamaki and Kawai, 1968; Jackson & Baker, 1970) and beeswax (Tulloch, 1970).

In vivo studies with radioactive precursors like acetate have demonstrated that several insects are capable of synthesizing hydrocarbons: *Dermestes vulpinus* (Clark & Bloch, 1959), *Musca domestica* (Robbins *et al.*, 1960), *Periplaneta americana* (Louloudes *et al.*, 1961; Nelson, 1969; Jackson & Baker, 1970), *Nezara viridula* (Gordon *et al.*, 1963), *Apis mellifera* (Piek, 1964), *Anthonomus grandis* (Lambremont *et al.*, 1966), *Oulema melanopus* (Lamb & Monroe, 1968) and *Heliothis zea* (Lambremont & Graves, 1969).

From *in vitro* experiments in the American cockroach, *P. americana*, it was evident that the integument was capable of hydrocarbon synthesis from 1-^{14}C-acetate and palmitate-1-^{14}C, whilst the fat body was not (Nelson, 1969).

Jackson & Baker (1970) provided evidence for incorporation of labelled fatty acids and several other precursors into the hydrocarbons of the same species, supplying the view of a direct or indirect relationship between fatty acid and hydrocarbon synthesis (Albro & Dittmer, 1970; Kolattukudy, 1970a, b). Hydrocarbon biosynthesis of arthropods other than insects has scarcely been reported. Zandee (1964, 1967) has provided data for the millipede *Graphidostreptus tumuliporus* and the spider *Avicularia avicularia*. After injection of the animals with 1-^{14}C-acetate the hydrocarbon fractions appeared to be highly radioactive, especially those from *Graphidostreptus*. In the study of Ross & Monroe (1970) results are given from the tarantula (*Aphonepelma* sp.) and the scorpion *Centruroides sculpturatus*.

The present paper reports the incorporation of the following precursors into the hydrocarbons of the millipede *G. tumuliporus*: 1-^{14}C-acetate, 1-^{14}C- and 16-^{14}C-palmitic acid, L-valine-^{14}C(U), L-isoleucine-^{14}C(U) and DL-mevalonic-2-^{14}C acid.

The results are presented for males and females separately, because important differences in fatty acid metabolism exist between both sexes of this species, as reported recently by us (Oudejans *et al.*, 1971a, b; Van der Horst *et al.*, 1972). The structure and composition of the hydrocarbon fractions will be dealt with in the next papers of this series (Oudejans, 1972).

MATERIALS AND METHODS

Some 200 specimens of the millipede *Graphidostreptus tumuliporus* (Karsch) were received from the "Institut Fondamental d'Afrique Noire" (I.F.A.N.), Dakar, Senegal in October 1970.

Radioactive precursors were purchased from New England Nuclear. Five males and five females were injected each with 10 μc Na-1-^{14}C-acetate (sp. act. 2 mc/mM); twenty-one males and six females each with 1·85 μc 1-^{14}C-palmitic acid (sp. act. 9·9 mc/mM); eighteen males and six females each with 2 μc 16-^{14}C-palmitic acid (sp. act. 51·2 mc/mM); fifteen females each with 3·33 μc L-isoleucine-^{14}C(U) (sp. act. 273 mc/mM); six males and nineteen females each with 2 μc L-valine-^{14}C(U) (sp. act. 219 mc/mM); twenty-two males and three females each with 2 μc DL-mevalonic-2-^{14}C acid (sp. act. 5·8 mc/mM).

Incubations with a supply of water but deprived of food lasted for 24 hr (in the acetate group for only 3 hr) at 24–25°C, and were ended by deep-freezing the animals at −26°C after anaesthetizing them with ether.

Lipids were extracted with chloroform–methanol as described earlier (Oudejans *et al.*, 1971a).

Hydrocarbons were obtained by chromatography of the unsaponifiable lipids on alumina (Merck) (Voogt, 1970) or by chromatography of parts of the total lipid on Florisil (Carroll, 1961).

Radioactivities of all fractions were determined with a Packard liquid scintillation spectrometer, Model 2420.

RESULTS

The quantities of the isolated total lipids and hydrocarbons are summarized in Table 1.

TABLE 1—QUANTITIES OF ISOLATED TOTAL LIPIDS AND HYDROCARBONS FROM THE DIFFERENT GROUPS OF *G. tumuliporus*

Precursor	No. of animals	Fresh weight (g)	Total lipids (% of fresh weight)	Hydrocarbons (% of lipids)
Na-1-^{14}C-acetate	5 males + 5 females	108·4	3·43	3·06
1-^{14}C-palmitic acid	21 males	180·6	2·02	2·94
	6 females	106·8	6·32	2·20
16-^{14}C-palmitic acid	18 males	132·9	2·30	2·93
	6 females	63·1	5·36	2·13
L-Isoleucine-^{14}C(U)	15 females	201·0	4·80	2·43
L-Valine-^{14}C(U)	6 males	52·3	2·24	3·08
	19 females	251·9	6·42	2·60
DL-Mevalonic-2-^{14}C-acid	22 males	157·5	2·44	2·93
	3 females	36·7	4·12	1·93

TABLE 2—RADIOACTIVITIES OF THE TOTAL LIPIDS AND HYDROCARBONS

		Total lipids		Hydrocarbons		
Precursor	Sex	Dis/min per mg	Percentage of injected dose	Dis/min per mg	Percentage of injected dose	Percentage of the total radioactivity in the lipids
Na-1-^{14}C-acetate	Male & Female	10,439	17·50	93,022	4·58	26·18
1-^{14}C-palmitic acid	Male	21,684	93·26	27,071	3·43	3·68
	Female	3822	98·19	2708	1·53	1·56
16-^{14}C-palmitic acid	Male	23,217	88·83	37,175	4·16	4·68
	Female	6802	86·27	7879	2·13	2·47
L-Isoleucine-^{14}C(U)	Female	102	0·89	194	0·04	4·50
L-Valine-^{14}C(U)	Male	400	1·76	613	0·08	4·55
	Female	102	1·96	87	0·04	2·04
DL-Mevalonic-2-^{14}C-acid	Male	2354	9·27	9746	1·12	12·08
	Female	1028	11·67	4419	0·97	8·31

The lipid content of the male specimens of *G. tumuliporus* ranged from 2·02 to 2·44 per cent of the fresh weight, whilst that of the females was between 4·12 and 6·42 per cent.

The rather variable lipid content of females can be explained from the fact that these animals often contain variable amounts of eggs, of which the lipids constitute as much as 14·45 per cent of the fresh weight (Van der Horst *et al.*, 1972).

Hydrocarbons constitute 2·93–3·08 and 1·93–2·60 per cent of the total lipids of males and females, respectively. Because of the variable glyceride content in the females the percentage of hydrocarbons is less constant than in the males. In Table 2 the radioactivities of the total lipids and hydrocarbons are shown expressed as specific radioactivity in dpm/mg and as the percentage of the total injected dose. Moreover, the radioactivities of the hydrocarbons are expressed as a percentage of the radioactivity incorporated into the total lipids.

From all precursors used incorporation of the label into the hydrocarbons occurred.

It is remarkable that in the group injected with 1-^{14}C-acetate more than one-quarter of the total lipid radioactivity is found in this fraction.

The interpretation of the values obtained from the 1-^{14}C- and 16-^{14}C-palmitic acid groups is more difficult. As palmitic acid is a lipid *per se* one will find nearly all radioactivity in the total lipids—even if no incorporation occurs—in contrast with other precursors from which lipids have to be synthesized.

Over 90 per cent of this injected acid appeared to be incorporated directly into the glycerides and phospholipids (Oudejans, unpublished data), by which the radioactivity in the hydrocarbons is not as high in comparison with the acetate group. In the mevalonic acid group some 10 per cent of the total lipid radioactivity occurred in the hydrocarbon fraction. Only in the animals injected with the labelled amino acids isoleucine and valine the incorporation into the total lipids and hydrocarbons is very low, although they are precursors of branched-chain fatty acids and hydrocarbons (Kaneda, 1967; Albro & Dittmer, 1969; Kolattukudy, 1970a).

DISCUSSION

Male as well as female specimens of the millipede *G. tumuliporus* are capable of synthesizing hydrocarbons from all precursors used (Table 2). The specific radioactivities of the hydrocarbons are in nearly all groups higher than those of the total lipids. After injection with 1-^{14}C-acetate the labelling of the hydrocarbon fraction is very high (more than 26 per cent of the radioactivity in the total lipids), especially if the low specific radioactivity of the injected acetate (2 mc/mM) is taken into account.

The data obtained from other arthropods agree with these findings, but usually the percentage of the label in the hydrocarbons is much lower (Louloudes *et al.*, 1961; Lambremont & Graves, 1969; Ross & Monroe, 1970).

Moreover, the incorporation of 1-^{14}C-acetate into the hydrocarbons of *Graphidostreptus* is a very fast process: even after an incubation period of only 15 min much of the radiolabel was found in the hydrocarbons (Oudejans, unpublished data). In order to determine the nondecarboxylative and decarboxylative mode of incorporation of fatty acids into hydrocarbons, 16-^{14}C- and 1-^{14}C-palmitic acid were used, respectively.

If the different doses and specific radioactivities of both precursors are taken into account, the specific radioactivity of the hydrocarbons of the 16-^{14}C-palmitic acid group would have been five times higher than that of the group with the carboxyl-labelled analogue, if direct incorporation into the hydrocarbons by a condensation mechanism without loss of the carboxyl carbon atom takes place.

In view of this it is clear from Table 2 that in the 1-^{14}C-palmitic acid group the incorporation is much higher than the expected value.

If there is only incorporation of acetyl-SCoA into the hydrocarbons a higher incorporation with 1-^{14}C-palmitic acid would have been expected.

However, it is unlikely to assume that the high radioactivity of the hydrocarbons in the 16-^{14}C-palmitic acid group has been arisen only from labelled acetyl-SCoA formed by β-oxidation. It is more probable that in this millipede a condensation as well as an elongation-decarboxylation mechanism are active in synthesizing hydrocarbons (Albro & Dittmer, 1970; Kolattukudy, 1970a, b).

Clear differences between 1-^{14}C- and 16-^{14}C-palmitic acid incorporation into hydrocarbons are reported by Jackson & Baker (1970) for *Periplaneta americana* and by Albro *et al.* (1970) for the microorganism *Sarcina lutea.*

Recently we reported also a high radioactivity in the hydrocarbons of the snail *Cepaea nemoralis* after injection of the animals with 16-^{14}C-palmitic acid (Van der Horst & Oudejans, 1972).

Incorporation of the branched amino acids into the hydrocarbons was very low (only 0·04–0·08 per cent of the injected dose), probably because most of the branched-chain hydrocarbons of *G. tumuliporus* possess an isoprenoid nature rather than an *iso* and *anteiso* type of branching.

Indeed, the results obtained from injection with mevalonic acid (3,5-dihydroxy-3-methyl valeric acid) as a precursor of isoprenoids point to a very rapid incorporation of this unit with 5 carbon atoms, although incorporation of some degradation products like acetate or other small moieties is possible.

Usually experiments with mevalonic acid are carried out in order to determine squalene and sterol biosynthesis or formation of triterpenoids. Some data on incorporation of mevalonic acid into the total hydrocarbon fractions of several molluscs are given by Voogt (1970). From arthropods only, Kaplanis *et al.* (1961) made mention of incorporation of this precursor into hydrocarbons of the house fly, *Musca domestica.*

From all these data it is obvious that in this millipede hydrocarbons are synthesized very rapidly and to a large extent. Hydrocarbon biosynthesis of this species will be investigated in more detail by *in vivo* and *in vitro* experiments.

Acknowledgements—The author wishes to thank Professor Dr. D. I. Zandee and Dr. D. J. van der Horst for their interest and for reading the manuscript.

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Key Word Index—Biosynthesis of hydrocarbons in a millipede; *Graphidostreptus tumuliporus*; millipede; Myriapoda; precursors of hydrocarbons; lipid biosynthesis in a millipede.