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HYDROCARBONS IN THE LAND SNAIL *CEPAEA NEMORALIS* (L.) (GASTROPODA, PULMONATA)

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Abstract—1. The biosynthesis of hydrocarbons in the snail *Cepaea nemoralis* was studied after injection of the ^{14}C-labelled precursors acetate, valine, isoleucine and palmitic acid.

2. The highest incorporation was achieved with palmitic acid, although with the other precursors the hydrocarbons were also distinctly labelled.

3. The composition of the hydrocarbons was determined by gas–liquid chromatography, involving a series of *n*-alkanes (n-C_{15}–n-C_{37}) as the major components (over 83 per cent).

INTRODUCTION

IN THE last decade many representatives of plants as well as animals have been studied for the occurrence and structure of hydrocarbons (reviews: Eglinton & Hamilton, 1967; Albro & Dittmer, 1970; Jackson & Baker, 1970; Kolattukudy, 1970a, b). The hydrocarbons of most species investigated constitute one of the major classes of lipids, especially in the cuticle. Apart from squalene (as an intermediate in sterol synthesis), no information to our knowledge has ever been presented about hydrocarbons in the phylum Mollusca, probably because from the molluscs investigated only very small hydrocarbon fractions have been isolated (Voogt, 1970), raising the question whether these hydrocarbons are significant endogenous metabolic products or perhaps just ingested with the food. However, in studies of the fatty acid metabolism of the land snail *Cepaea nemoralis* (L.) (van der Horst, 1970) attention was drawn to the hydrocarbon fraction because this snail appeared to incorporate several radioactive precursors into the hydrocarbons. Besides, in the literature evidence is presented for a relationship between fatty acid and hydrocarbon synthesis (reviews: Albro & Dittmer, 1970; Jackson & Baker, 1970; Kolattukudy, 1970a, b).

In this paper the composition of the hydrocarbons of *C. nemoralis* is given, whilst biosynthesis of these products is reported with the precursors acetate, valine, isoleucine and palmitic acid.

MATERIALS AND METHODS

Four different groups of snails of the species *Cepaea nemoralis* (L.) (Mollusca, Gastropoda, Pulmonata, Stylommatophora) were collected in the vicinity of Utrecht, except for group 1, of which most of the animals have been collected near Groningen. In a series of

experiments radioactive precursors were administered to the animals by injection into the foot. The most important data are listed in Table 1.

Incubations lasted for 48 hr under the conditions described formerly (van der Horst, 1970) and were ended by deep-freezing. Lipids were extracted according to van der Horst *et al.* (1969). From all groups hydrocarbon fractions were obtained by chromatography of the unsaponifiable lipids on an alumina (Merck) column (Voogt, 1970), except in the more comprehensive experiments in the first group, from which hydrocarbons were obtained equally well without prior saponification. In the latter case part of the total lipid was divided into a phospholipid and a neutral lipid fraction (Lipsky *et al.*, 1957) and the neutral lipid fractionated on a Florisil column according to Carroll (1961).

TABLE 1—DATA CONCERNING THE DIFFERENT GROUPS OF *C. nemoralis* USED AND THE RADIOACTIVE PRECURSORS ADMINISTERED

	Group			
	1	2	3	4
No. of animals used	350	12	25	36
Fresh weight without shells (g)	584	24·5	43·2	61·4
Collecting date	June 1970	October 1970	February 1971	February 1971
Precursor	Na-1-^{14}C-acetate	L-Valine-^{14}C (U)	L-Isoleucine-^{14}C (U)	16-^{14}C-palmitic acid
Specific radioactivity mC_i/mM	2	219	273	51
Dose (μC_i/animal)	1	1	1	1·25

Reagent grade solvents were used. Hexane was redistilled over potassium permanganate and the fraction boiling at 67–68°C was collected (Hanahan *et al.*, 1963).

Precautions were taken to avoid any contamination of the lipid samples with dust hydrocarbons (Gelpi *et al.*, 1970). Parts of the hydrocarbon fractions were hydrogenated in redistilled iso-octane according to Louloudes *et al.* (1962). Analytical gas–liquid chromatography of the total and hydrogenated samples was performed on a Becker 2300 instrument equipped with dual flame ionization detection, using two different stationary phases:

SE-52 (4%) coated on acid-washed Chromosorb W (100–120 mesh), precoated with 5% dimethyldichlorosilane (Horning *et al.*, 1959), packed in an aluminium column (1·80 m × 4 mm i.d.); 20% polyethyleneglycoladipate (PEGA) + 3% phosphoric acid coated on acid-washed Chromosorb W (60–80 mesh), packed in an aluminium column with the same dimensions.

Nitrogen was used as carrier gas, outlet flow was 50 ml/min. Column temperature was 170°C.

A semilogarithmic plot of retention times against carbon number of the separated components was used for identification (James, 1960).

The following standard hydrocarbons were available: n-C_{12}–n-C_{36} were purchased from Applied Science Laboratories; anteiso-C_{20}, anteiso-C_{26}, iso-C_{21} and iso-C_{29} were a gift of Dr. S. A. Ballard (Koninklijke/Shell-Laboratorium, Amsterdam); iso-C_{25}, iso-C_{31}, anteiso-C_{25} and a mixture of the iso and anteiso-branched hydrocarbons from tobacco were donated by Dr. J. D. Mold (Liggett & Myers Tobacco Co., Durham, N.C.).

Quantitative compositions of the hydrocarbon fractions were calculated from the peak areas on the chromatograms.

Radioactivities of all fractions were measured in toluene-Omnifluor (New England Nuclear) with a Packard liquid scintillation spectrometer, Model 2420.

RESULTS

The content of hydrocarbons in the various groups of *C. nemoralis* was quite low and ranged between 0·46–0·54 per cent of the total lipids. As the total lipid content in *Cepaea* is only 1·25–1·40 per cent of the fresh weight (van der Horst, 1970) only a few milligrams of hydrocarbons were obtained in the various isolation procedures. Radioactivities of the lipid fractions are summarized in Table 2.

The compositions of the isolated hydrocarbon fractions were determined. No notable differences were observed between the various groups and in the acetate group the content and composition of the hydrocarbons was not influenced by the application of two different isolation procedures. The hydrocarbon mixtures appeared to be fully saturated as the fractions did not change upon hydrogenation. Over 83 per cent of the hydrocarbons consisted of *n*-alkanes. Furthermore, minor branched-chain components of at least three types of branching could be detected.

TABLE 2—RADIOACTIVITIES OF THE TOTAL LIPIDS AND HYDROCARBONS OF THE DIFFERENT GROUPS OF *C. nemoralis*

	Group			
	1 Acetate	2 Valine	3 Isoleucine	4 Palmitic acid
Specific radioactivity total lipids (dis/min per mg)	2729·0	393·4	429·0	84,676·0
Specific radioactivity hydrocarbons (dis/min per mg)	164·2* 163·5†	144·0	57·0	16,784·8

* After saponification of the total lipids.
† After chromatography of the neutral lipids on Florisil.

On the SE-52 column three parallel lines resulted by plotting the logarithm of the retention times of the various components against a linear ordinate, representing the number of carbon atoms present in the hydrocarbons: one of the *n*-alkanes, a coincidental iso-anteiso line and a line of an unknown branched-type hydrocarbons which were eluted even before the iso and anteiso-branched ones.

On the PEGA column iso- and anteiso-branched hydrocarbons were separated, but unfortunately with this type of column the iso-branched ones were eluted together with the unknown branched-type hydrocarbons of the consecutive carbon number, so in this plot again three parallel lines resulted. The combined results are summarized in Table 3.

TABLE 3—COMPOSITION OF THE HYDROCARBONS (IN moles per cent) OF *C. nemoralis* AS DETERMINED BY GLC ON TWO DIFFERENT STATIONARY PHASES

C-number	*n*-alkanes	2-Methyl alkanes (iso)	3-Methyl alkanes (anteiso)	Unknown branched alkanes
15	0·11	—	—	—
16	0·32	—	—	0·07
17	0·47	—	—	0·13
18	0·64	—	0·24	—
19	0·78	—	0·30	0·27
20	1·20	0·30*		0·75
21	1·86	—	0·50	0·74
22	2·80	0·25*		0·34
23	3·98	0·16	0·14	0·46
24	5·77	0·20	—	0·26
25	6·22	0·50	—	0·41
26	8·08	0·28	—	0·57
27	7·89	1·61	—	0·52
28	8·40	0·65	—	0·47
29	6·98	0·88	—	0·22
30	6·50	0·47*		0·49
31	6·23	0·52*		0·11
32	4·33	0·59*		0·66
33	3·75	0·05*		0·20
34	2·70	0·21*		0·61
35	2·30	0·40*		0·60
36	1·71	0·50*		0·31
37	Trace			
	83·02	8·75*		8·19

* Iso + anteiso.

DISCUSSION

C. nemoralis is capable of synthesizing hydrocarbons from all precursors used (Table 2). The remarkably high specific radioactivity of the hydrocarbons after injection with 16-^{14}C-palmitic acid supports the view that fatty acids are intermediates in the synthesis of hydrocarbons. Whether a condensation or an elongation–decarboxylation mechanism is favoured cannot be decided from these experiments. Valine and isoleucine are considered as precursors of iso- and anteiso-branched fatty acids and hydrocarbons (Kaneda, 1967; Albro & Dittmer, 1969; Kolattukudy, 1970a). Although degradation of the injected amino acids to lower metabolic units (e.g. acetyl-SCoA and succinyl-SCoA) (Grigor *et al.*, 1970) and incorporation of these units into the hydrocarbons cannot be ruled out, from the high radioactivity of the hydrocarbons of the valine and isoleucine groups in comparison with those of the acetate group it is very plausible that direct incorporation of these branched-chain amino acids into the branched-chain

hydrocarbons takes place. In this connexion it is remarkable that in *C. nemoralis* no iso-branched fatty acids occur (van der Horst, 1970).

The biosynthesis of hydrocarbons from acetate is rather low in comparison with other animals studied in this field, for instance, the millipede *Graphidostreptus tumuliporus* (Oudejans, 1972), the beetle *Dermestes vulpinus* (Clark & Bloch, 1959), the housefly, *Musca domestica* (Robbins *et al.*, 1960), the American cockroach, *Periplaneta americana* (Louloudes *et al.*, 1961) and the boll weevil, *Anthonomus grandis* (Lambremont *et al.*, 1966).

The composition of the hydrocarbons of *C. nemoralis* is different from those of other animals, bacteria or plants.

In most animals studied until now one or a restricted number of hydrocarbons is predominant, viz. C_{27}-diene in *P. americana* (Beatty & Gilby, 1969) or C_{27} (straight-chain and methyl-branched) in some cockroaches (Tartivita & Jackson, 1970).

In plant hydrocarbons a predominance of odd-chain *n*-alkanes is overwhelming. Very often 90 per cent or more of the paraffin fraction is C_{29} or C_{31} (Kolattukudy, 1970a).

Bacterial hydrocarbons are rather complex with great differences between the species. In *Sarcina lutea* the major hydrocarbon is a C_{29} di-branched one (Albro & Dittmer, 1970).

In *C. nemoralis* there is no predominance of odd-chain *n*-alkanes over even-chain ones. The percentages of the spectrum of *n*-alkanes starting from pentadecane (*n*-C_{15}) are regularly ascending to a maximum (octacosane, *n*-C_{28}) and descending slowly to heptatriacontane (*n*-C_{37}). Of the branched-chain hydrocarbons 2-methylhexacosane (iso-C_{27}) is the predominant one (1·61%).

In our experiments we failed to detect squalene in the hydrocarbon fractions, although *Cepaea* is quite capable of synthesizing sterols (van der Horst & Voogt, 1972). These findings resemble those of another snail of the same family, *Arianta arbustorum*, in which also no traces of squalene could be detected (van der Horst, unpublished results) and from which very high labelled sterols have been isolated (Voogt & van der Horst, 1972).

The unknown branched-chain hydrocarbons will be reinvestigated when a combined gas chromatograph–mass spectrometer is available.

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REFERENCES

ALBRO P. W. & DITTMER J. C. (1969) The biochemistry of long-chain, nonisoprenoid hydrocarbons—II. The incorporation of acetate and the aliphatic chains of isoleucine and valine into fatty acids and hydrocarbons by *Sarcina lutea in vivo*. *Biochemistry* **8**, 953–959.

ALBRO P. W. & DITTMER J. C. (1970) Bacterial hydrocarbons: occurrence, structure and metabolism. *Lipids* **5**, 320–325.

BEATTY I. M. & GILBY A. R. (1969) Major hydrocarbon of a cockroach cuticular wax. *Naturwiss.* **56**, 373.

CARROLL K. K. (1961) Separation of lipid classes by chromatography on Florisil. *J. Lipid Res.* **2**, 135–141.

CLARK A. J. & BLOCH K. (1959) The absence of sterol synthesis in insects. *J. biol. Chem.* **234**, 2578–2582.

EGLINTON G. & HAMILTON R. J. (1967) Leaf epicuticular waxes. *Science* **156**, 1322–1335.

GELPI E., NOONER D. W. & ORÓ J. (1970) The ubiquity of hydrocarbons in nature: aliphatic hydrocarbons in dust samples. *Geochim. Cosmochim. Acta* **34**, 421–425.

GRIGOR M. R., DUNCKLEY G. G. & PURVES H. D. (1970) The synthesis of the branched-chain fatty acids of rat skin surface lipid. *Biochim. biophys. Acta* **218**, 389–399.

HANAHAN D. J., EKHOLM J. & JACKSON C. M. (1963) Studies on the structure of glyceryl ethers and the glyceryl ether phospholipids of bovine erythrocytes. *Biochemistry* **2**, 630–646.

HORNING E. C., MOSCATELLI E. A. & SWEELEY C. C. (1959) Polyester liquid phases in gas–liquid chromatography. *Chemy Ind.* **1959**, 751–752.

JACKSON L. L. & BAKER G. L. (1970) Cuticular lipids of insects. *Lipids* **5**, 239–246.

JAMES A. T. (1960) Qualitative and quantitative determination of fatty acids by gas chromatography. In *Methods of Biochemical Analysis* (Edited by GLICK D.), pp. 1–59. Interscience, New York.

KANEDA T. (1967) Biosynthesis of long chain hydrocarbons—I. Incorporation of L-valine, L-threonine, L-isoleucine and L-leucine into specific branched-chain hydrocarbons in tobacco. *Biochemistry* **6**, 2023–2032.

KOLATTUKUDY P. E. (1970a) Plant waxes. *Lipids* **5**, 259–275.

KOLATTUKUDY P. E. (1970b) Biosynthesis of cuticular lipids. *Ann. Rev. Plant Physiol.* **21**, 163–192.

LAMBREMONT E. N., BUMGARNER J. E. & BENNETT A. F. (1966) Lipid biosynthesis in the boll weevil (*Anthonomus grandis* Boheman) (Coleoptera: Curculionidae). Distribution of radioactivity in the principal lipid classes synthesized from ^{14}C-1-acetate. *Comp. Biochem. Physiol.* **19**, 417–429.

LIPSKY S. R., HAAVIK A., HOPPER C. L. & MCDIVITT R. W. (1957) The biosynthesis of fatty acids in the plasma of man—I. The formation of certain chromatographically separated higher fatty acids of the major lipid complexes from acetate-1-^{14}C. *J. Clin. Invest.* **36**, 233–244.

LOULOUDES S. J., CHAMBERS D. L., MOYER D. B. & STARKEY J. H., III (1962) The hydrocarbons of adult houseflies. *Ann. ent. Soc. Am.* **55**, 442–448.

LOULOUDES S. J., KAPLANIS J. N., ROBBINS W. E. & MONROE R. E. (1961) Lipogenesis from C^{14}-acetate by the American cockroach. *Ann. ent. Soc. Am.* **54**, 99–103.

OUDEJANS R. C. H. M. (1972) Hydrocarbons in the millipede *Graphidostreptus tumuliporus* (Karsch) (Myriapoda: Diplopoda)—I. *In vivo* incorporation of ^{14}C-labeled precursors into the hydrocarbon fraction. *Comp. Biochem. Physiol.* (in press.)

ROBBINS W. E., KAPLANIS J. N., LOULOUDES S. J. & MONROE R. E. (1960) Utilization of 1-C^{14}-acetate in lipid synthesis by adult house flies. *Ann. ent. Soc. Am.* **53**, 128–129.

TARTIVITA K. & JACKSON L. L. (1970) Cuticular lipids of—I. Hydrocarbons of *Leucophaea maderae* and *Blatta orientalis*. *Lipids* **5**, 35–37.

VAN DER HORST D. J. (1970) Investigation of the synthesis and distribution of fatty acids in the lipids of the snail *Cepaea nemoralis* (L.)—I. The fatty acid composition of the total lipids. *Neth. J. Zool.* **20**, 433–444.

VAN DER HORST D. J., VAN GENNIP A. H. & VOOGT P. A. (1969) A simplified method for extracting lipids from large quantities of tissue abundant in water. *Lipids* **4**, 300–301.

VAN DER HORST D. J. & VOOGT P. A. (1972) Biosynthesis and composition of sterols and sterol esters in the land snail *Cepaea nemoralis* (L.) (Gastropoda, Pulmonata, Stylommatophora). *Comp. Biochem. Physiol.* (in press.)

VOOGT P. A. (1970) Onderzoek naar het vermogen tot sterolsynthese en de samenstelling van de sterolen in het phylum Mollusca. Thesis, University of Utrecht.
VOOGT P. A. & VAN DER HORST D. J. (1972) Investigations of the capacity of synthesizing 3β-sterols in Mollusca—VIII. The biosynthesis of 3β-sterols in the pulmonates *Succinea putris* and *Arianta arbustorum*. *Archs. int. Physiol. Biochim.* (in press.)
malonyl-CoA incorporated into long-chain fatty acids/min per mg protein. The

Key Word Index—Hydrocarbons in a snail; *Cepaea nemoralis* (snail); biosynthesis of hydrocarbons; lipid metabolism in a snail.