Crustacean Surface Waxes. Part I. The Hydrocarbons from the Surface of *Ligia oceanica*

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The surface lipids of the crustacean *Ligia oceanica* have been isolated, and the hydrocarbons separated into six major fractions. The n-alkanes, with a maximum chain length at C_{33} and a minimum at C_{14}, are the largest fraction (3·85%): the others are 2,6,10,14-tetramethylpentadecane (pristane) (0·82%), heptadec-8-ene (0·55%), heptadeca-8,13-diene (0·45%), two unknown hydrocarbons (0·65%), and cholestadiene (0·3%), the last identified for the first time in marine lipids.

THE crustacean *Ligia oceanica* is a member of the order Isopoda, sub-class Malacostraca in the Phylum Arthropoda. It is related to the terrestrial wood louse (Slater) *Porcellio scaber* and is sometimes called the shore slater. *L. oceanica* is restricted to the seashore around the British Isles, inhabiting rocky beaches above high tide marks. Occupying the middle ground between the terrestrial and the marine environments the shore slater represents a type of crustacean whose chemical composition has not been examined to any great extent.

Like terrestrial arthropods, *Ligia oceanica* has a layer of wax over the cuticle as its primary adaptation towards waterproofing. It has been suggested the cuticle is poorly developed because the creature inhabits a damp environment.[1]

Since the wood louse is reported to [2] contain a series of unusual alcohols, *e.g.* slaterol, it was decided to investigate the surface constituents of *Ligia oceanica*, in order to compare the composition and character of the wax with those of other arthropods and other marine species.

[1] E. B. Edney, *J. Exp. Biol.*, 1951, **28**, 91.
[2] G. W. K. Cavill, D. V. Clark, and H. Hinterberger, *Austral. J. Chem.*, 1966, **19**, 1495.

RESULTS AND DISCUSSION

The first fraction from the surface lipids of *L. oceanica* isolated by column chromatography and purified by t.l.c. showed i.r. absorption characteristic of hydrocarbons, *viz.* $\nu_{max.}$ 3020 (alkene C–H), 2940 (CH_2 stretch), 2860 (CH_3 stretch), 1460 (CH_2 bend), and 1380 cm^{-1} (CH_3 bend). The u.v. spectrum ($\lambda_{max.}$ 206, 216, and 236 nm) indicated the presence of a conjugated heteroannular double bond system. G.l.c. of the total mixture showed a range of hydrocarbons with a maximum chain length at C_{33} and a minimum at C_{14} (Table).

T.l.c. on silver nitrate-impregnated silica gel separated the hydrocarbon fraction into five discrete bands which corresponded in R_F values to saturated hydrocarbons (78%), monounsaturated hydrocarbons (8%), cholestadiene (4%), a diunsaturated hydrocarbon (7%), and other material (2%). Material from each of these bands was isolated by preparative t.l.c.

The saturated hydrocarbons consisted of a mixture ranging in chain length from C_{14} to C_{33}, with the hydrocarbon of retention index [3] 1700 as the major component (Table). These could be further separated by using their differing abilities to undergo clathration with Linde molecular sieve.[4] The branched chain hydrocarbons (Table) were not clathrated by the sieve; their major components have retention indices (R.I.) of 1700 (60%) and 1800 (20%). The major branched component (R.I. 1700) was subjected to g.l.c.–mass spectrometry: the fragmentation pattern was very similar to that of pristane,[5] showing peaks at *m/e* 268 (M^+), $M - 15$, and $M - 43$, with peaks at *m/e* 183 and 113 characteristic of cleavage at the internal methyl branches. The other major constituent (R.I. 1800) is almost certainly phytane, although this material was not subjected to g.l.c.–mass spectrometry.

Composition (%) of the hydrocarbon fractions of *Ligia oceanica* *

Retention index	Total saturated hydrocarbons	Total unsaturated hydrocarbons	Branched-chain hydrocarbons †	Saturated straight-chain hydrocarbons
1400	0·2			0·3
1450	0·4			0·5
1500	8·1	1·0	5·0	8·8
1550		1·3	2·0	
1600	1·5	0·6	4·0	0·5
1650	0·3	0·2	5·0	0·4
1700	16·5	82·0	60·0	0·1
1750	Trace		4·0	
1800	7·3	5·9	20·0	0·2
1850	Trace			
1900	4·2	9·0		5·7
1950	Trace			
2000	2·9			3·8
2050	0·4			0·5
2100	4·1			5·5
2200	5·7			7·7
2300	5·9			8·0
2400	4·4			5·9
2500	4·9			6·7
2600	5·8			7·8
2700	5·8			7·7
2800	5·2			7·1
2900	5·5			7·5
3000	2·8			3·7
3100	4·2			5·7
3200	2·2			3·0
3300	1·7			2·3

* G.l.c. analyses on 1% OV17 by temperature programming on a Pye 104 dual field ionisation detector instrument with 5 ft × ¼ in columns.

In the marine environment, phytane has been found in many organisms from shark liver oil [6] to marine zooplankton.[7] It is believed that most of the observed pristane is formed from phytol by copepods. *Ligia oceanica* is omniverous but it eats chiefly *Fucus vesiculosus*, a member of the brown algae family. In a recent report [8] on the unsaponifiable components of *Fucus vesiculosus* the presence of pristane was noted, but its proportion was not recorded. It is presumably from this food source that *L. oceanica* obtains the pristane or its precursor phytol.

The proportion of pristane (0·82%) in this extract is unusually high for most natural lipid mixtures, suggesting that either the biochemical conversion of phytol into pristane is very efficient in this crustacean, or the mechanisms for breakdown of pristane are less effective than in most marine organisms.

The ratio of pristane to phytane is 3 : 1, which can be contrasted with the ratios found by Ackman [9] of 1 : 2 for fresh water fish and 170 : 1 for sand launce, a salt water fish. No value for this ratio in *Fucus vesiculosus* is given.[8] The principal difference between these results for *L. oceanica* and those for members of the Insecta is the presence of non-isoprenoid methyl branched hydrocarbons in cockroaches [10] but the absence of pristane and phytane. It is clear that as far as the branched and straight chain hydrocarbons are concerned comparison must be made with the marine environment rather than the terrestrial one for *Ligia oceanica*.

The monoalkene fraction (7% of the total hydrocarbons) was isolated by t.l.c. (silver nitrate-impregnated silica gel) and showed u.v. absorption at 206 nm. The major constituent (Table) had a g.l.c. retention index of 1700. Its mass spectrum showed a parent ion at *m/e* 238 ($C_{17}H_{34}$) and the remainder of the spectrum showed the familiar progression of peaks $C_nH_{2n-1}^+$ indicating that this heptadecene was a straight chain alkene. The position of the double bond was determined by a modified von Rudloff technique.[11] The free acids consisted of octanoic and nonanoic acids, showing that the alkene was heptadec-1-ene.

By similar techniques the diunsaturated hydrocarbon fraction was shown to consist mainly of one component with R.I. 1700 on OV17 and 1800 on diethylene glycol

[3] E. Kovats, *Helv. Chim. Acta*, 1958, **41**, 1915.
[4] R. J. Hamilton, Ph.D. Thesis, 1962.
[5] R. J. Hamilton, M. Long, and M. Y. Raie, *J. Amer. Oil Chemists' Soc.*, 1972, **49**, 307.
[6] E. Gelpi and J. Oro, *J. Amer. Oil Chemists' Soc.*, 1968, **45**, 144.
[7] M. Blumer and D. W. Thomas, *Science*, 1965, **148**, 370.
[8] T. G. Halsall and I. R. Hills, *Chem. Comm.*, 1971, 448.
[9] R. G. Ackman, *Lipids*, 1971, **6**, 520.
[10] L. L. Jackson, *Lipids*, 1969, **5**, 38.
[11] R. J. Hamilton and M. Y. Raie, *Chem. and Ind.*, 1971, 1228.

succinate. G.l.c.–mass spectrometry confirmed the molecular formula as $C_{17}H_{32}$. The mass spectrum was characteristic of alkadienes, showing fragments due to allylic fission and to molecular rearrangement. The products of oxidation were nonanoic and butanoic acids, suggesting that the hydrocarbon was heptadeca-4,8-diene. The occurrence of alkenes and alkadienes in the Isopoda has not been reported, although zamene (a branched C_{19} monoalkene) has been found in marine zooplankton,[7] heptacosa-6,9-diene in *Periplaneta americana* (cockroach) lipids,[12] and tricos-9-ene in *Periplaneta australasiae*.

The mechanisms of biosynthesis of insect waxes are not clearly understood. In some insects,[13] the hydrocarbons have been shown to be biosynthesised from long-chain fatty acids. If these C_{17} hydrocarbons are biosynthesised by *L. oceanica*, one would expect that the fatty acid precursor of heptadec-8-ene would be oleic acid (which seems possible) but heptadeca-4,8-diene would require either octadeca-9,13-dienoic acid or octadeca-4,8-dienoic acid (neither of which has been identified in *L. oceanica*). Of the two, octadeca-9,13-dienoic acid seems the more likely. If, however, the hydrocarbons or their fatty acid precursors are derived directly from foodstuffs, one should expect to find some evidence of them in *Fucus vesiculosus*. The fatty acids of *F. vesiculosus* contain octadeca-9,12-dienoic [14] acid in addition to some C_{20} and C_{22} tetra- and penta-enoic acids. Thus linoleic acid could only act as the precursor to the *L. oceanica* hydrocarbons if one of the double bonds had migrated to the 9,13-position. The hydrocarbons which have been found in *Fucus vesiculosus* include heneicosa-1,6,9,12,15,18-hexaene and heneicosa-1,6,9,12,15-pentaene, whose precursors are the C_{22} polyunsaturated acids which have been reported in the algae in the most recent survey. Further study is therefore required to determine whether *L. oceanica* biosynthesises hepta-4,8-diene or absorbs it from its food.

A further fraction (R_F 0·69 in $AgNO_3$–SiO_2 t.l.c.) was analysed by g.l.c., which showed that it was a mixture of components whose chain lengths ranged from C_{31} to C_{37}, with C_{35} as the major component. No further information was obtained for this fraction.

The remaining fraction showed strong u.v. absorption at 234 nm suggesting a heteroannular diene system. Analysis by g.l.c. on 1% OV17 at 260 °C revealed the presence of a single component, R.I. 2980. G.l.c.–mass spectrometry showed a peak at *m/e* 368 (both parent and base), consistent with cholestadiene ($C_{27}H_{44}$). Major peaks were also observed at *m/e* 353 (*M* — Me) and 255 (loss of side chain). Tetracyclic triterpenes cleave across ring D to give a fragment containing rings A, B, and C (*m/e* 214), which breaks down to a fragment $C_{12}H_{25}$ (*m/e* 159); both these ions were present in the spectrum. These two peaks at *m/e* values 4 mass units less than the corresponding cholestane fragments,[15] in addition to a peak at *m/e* 53, suggested that the molecule was cholesta-3,5-diene. Finally, analysis of cholesteryl tosylate on a g.l.c. column at 260°, leading to its decomposition and the elution of cholesta-3,5-diene [16] (R.I. 2980), confirmed that the component in *L. oceanica* was 3,5-cholestadiene.

Cholesterol is the major sterol of *L. oceanica* [17] and occurs in both free and esterified forms. The presence of cholestadiene may indicate the presence of an enzyme capable of dehydrating the sterol. The reason for the requirement for such a molecule in the surface wax is open to conjecture, but it may be that the cyclic compound, although high in molecular weight, will prevent the surface wax from crystallising in the wrong shape for its protecting properties.

The evidence thus far presented shows that the cuticular wax of *Ligia oceanica* is well-defined, and not poorly developed as was previously suggested.

EXPERIMENTAL

The specimens of *Ligia oceanica* were collected at the Marine Biology Station, Mill Port, Cumbrae, Scotland.

All solvents used were of AnalaR grade. I.r. spectra were determined (solvent carbon tetrachloride) with a Unicam SP 1200 grating spectrophotometer. U.v. spectra were determined with a Unicam SP 1800 spectrophotometer. An LKB 9000 g.l.c.–mass spectrometer was used for the mass spectral determinations. G.l.c. analyses were performed on a Pye 104 dual field ionisation detector instrument.

Extraction of Lipids.—The surface lipids were extracted by dipping the bodies of *L. oceanica* (500) in light petroleum (b.p. 40—60°) (1 dm^3) for 15 min. After removal of the solvent, the bodies were immersed in a fresh portion of light petroleum (1 dm^3) for 24 h. After removal of this solvent, the bodies were homogenised in light petroleum (1 dm^3) for 15 min to obtain a further lipid extract. In each case, the lipid material, which had a light brown colour, was obtained by decantation, filtration, and removal of the solvent by distillation. The extract was blanketed in nitrogen and stored in a refrigerator.

Column Chromatography.—The surface lipid (1·31 g) was placed on a column of silica gel (50 g; 0·2—0·5 mesh) in light petroleum (b.p. 40—60°) (10 cm^3). Elution with light petroleum yielded the hydrocarbons (85 mg).

Thin-layer Chromatography.—Silver nitrate-impregnated silica gel plates (5) loaded with hydrocarbons (100 mg) were developed in light petroleum (b.p. 40—60°)–diethyl ether (19 : 1 v/v). Components were located by spraying with 2,7-dichlorofluorescein. The bands were scraped from the plates into sintered filters and extracted with either cold or warm chloroform (100 cm^3). This removed any non-hydrocarbon impurities from the column chromatographic separation. R_F Values were: for saturated hydrocarbons 0·86, monounsaturated 0·74, cholestadiene 0·71, unknown 0·69, and diunsaturated 0·55.

Separation of Unsaturated Hydrocarbons.—The hydrocarbons (92·0 mg) were separated on t.l.c. plates ($AgNO_3$–

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[13] R. D. Nelson, *Nature*, 1969, **221**, 854.
[14] G. R. Jamieson and E. H. Reid, *Phytochemistry*, 1972, **11**, 1423.
[15] H. Budzkiewicz, J. M. Wilson, and C. Djerassi, *J. Amer. Chem. Soc.*, 1963, **85**, 3688.
[16] W. J. A. Vanden Heuvel, *J. Chromatog.*, 1967, **26**, 396.
[17] M. Y. Raie, Ph.D. Thesis, 1973.

SiO_2) with light petroleum (b.p. 40—60°) as eluant, giving a saturated fraction (70·0 mg), a monounsaturated fraction (5·6 mg), and a diunsaturated fraction (5·0 mg). Two further spots on the t.l.c. plate had R_F 0·74 (2·0 mg), and 0·71 (0·4 mg).

Separation of Branched Hydrocarbons.—The saturated hydrocarbons (23 mg) were dissolved in iso-octane (10 cm^3) and added to Linde 5 Å molecular sieve (1·3 g). The mixture was refluxed for 100 h, cooled, and filtered. The filtrate contained the branched and cyclic alkanes (6 mg). The saturated hydrocarbons which had been clathrated by the molecular sieve were liberated by digestion of the sieve with hydrofluoric acid. Dilution and neutralisation of the hydrofluoric acid solution followed by extraction with ether yielded the saturated hydrocarbons (17 mg).

Preparation of Cholesteryl Tosylate.—Cholesterol (1·4 g) and toluene-*p*-sulphonyl chloride (0·1 g) were dissolved in ethyl acetate (100 cm^3) and refluxed overnight. The tosyl derivative was analysed by g.l.c. on a 1% OV17 column (7 ft × ¼ in) at 270 °C; it decomposed on the column to give cholestadiene.

Oxidation of the Unsaturated Hydrocarbons.—The C_{17} alkadiene (1 mg) was dissolved in t-butyl alcohol (250 μl) and the von Rudloff oxidation mixture (500 μl) was added. After 1 h at room temperature the mixture (50 μl) was injected onto a Porapak Q column (15 ft × ¼ in) for analysis of the liberated free fatty acids.

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