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Unsaponifiable Constituents of Blowfly Larvae (*Calliphora erythrocephala*)

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The initial object of the present work was to find out whether ubiquinone (coenzyme Q) occurred in the insect world, and, if it did, to identify the particular isoprenologue. The larval form of the blowfly was chosen as an easily-accessible raw material obtainable from firms who sell the 'gentles' as bait to anglers. To attain the initial goal it was necessary to prepare the total unsaponifiable fraction and to carry out a separation of the various constituents. In so doing the scope of the investigation was widened.

MATERIALS AND METHODS

Blowfly larvae. These were bought from the 'gentles' farm of Ellis Ashurst Ltd., Conisburgh, Yorks. They arrived packed in sawdust and shavings and were too active to be easily picked out. The container and its contents were therefore cooled to -30° for about 1 hr. so that the dead larvae and sawdust could be more readily separated.

Saponification. The larvae (free from sawdust) were homogenized in a mechanical blender. Aq. KOH [60% (w/v); 0.5 ml./g. of tissue] and methanolic pyrogallol (0.3%; 1 ml./g. of tissue) were mixed and then added to the homogenate. The mixture was heated under reflux for 1 hr. on a water bath (Mervyn & Morton, 1959; Mervyn, 1957).

After being cooled, the solution was diluted with water (2 vol.) and extracted five times with diethyl ether (freshly redistilled from reduced iron). The combined extracts were

washed until free from alkali and dried (over Na_2SO_4), and the ether was removed under nitrogen. The unsaponifiable matter, and fractions derived from it, were stored in the dark at -20° as far as possible.

Alumina. Grade O alumina (P. Spence and Co., Widnes, Lancs.) was acid-washed, heated to 400° and partially deactivated to Brockmann Grade 3 by addition of water to a slurry made with light petroleum (b.p. $40-60^{\circ}$).

Adsorption chromatography. The unsaponifiable material was chromatographed first on alumina and some fractions were re-chromatographed on magnesia (Laidman, Morton, Paterson & Pennock, 1960). Details are shown in Table 1.

Spectroscopy. All the fractions were examined in cyclohexane in the visible and ultraviolet regions (in a Unicam SP. 500 instrument), and infrared spectra of some fractions were measured (in the Infracord spectrophotometer of Perkin-Elmer) either as films or in potassium bromide disks.

Reversed-phase chromatography on paper. The system petroleum jelly-treated paper (stationary phase)-2.5% (v/v) water in dimethylformamide (mobile phase) (Linn *et al.* 1959) was used, and pure specimens of ubiquinones were used for calibration purposes. The method was made semiquantitative by extracting separated isoprenologues from sections of paper after development, followed by ultraviolet spectrophotometry on the extracts.

Determination of ubiquinone. Provided that the absorption curve showed the absence of significant irrelevant absorption the molar concentration could be determined directly by measuring the intensity of absorption at $272 \text{ m}\mu$ in cyclohexane. When there was too much irrelevant absorption for this procedure to be valid, the quinone was reduced by KBH_4 , and the change in E at $272 \text{ m}\mu$ was measured (Crane, Lester, Widmer & Hatefi, 1959).

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Table 1. *Chromatographic fractions obtained from unsaponifiable matter of blowfly larvae*

From 420 g. of larvae was obtained 1.12 g. of unsaponifiable matter, which was chromatographed on alumina (Brockmann 3, acid-washed; 100 g.).

Fraction	Eluent		Wt. of fraction (mg.)	Appearance	Spectroscopic properties	
	Vol. (l.)	Composition [% ether in light petroleum (b.p. 40–60°)]			$\lambda_{\text{max.}}$ (m μ)	$E_{1\text{ cm.}}^{1\%}$
1	1.0	2	316	Colourless oil	232	31.3
	0.5	4				
2	0.5	4	26.3	Orange-coloured oil	272	95
	0.5	6				
3	0.5	6	32.3	Colourless oil	232*	34.8
	1.0	10			265*	15.5
4	1.0	25	339	White solid	262*	14.5
					272	20.9
					282	23.4
					295	17.9
					310*	8.4
5	1.0	100	11.5	Colourless oil	230*	64.5
					283*	37.0
					296	38.8
					307*	36.5
6	0.5	Methylal	23.6	Orange-coloured oil	232	41.5

* Inflection.

EXPERIMENTAL

Table 1 refers to a definitive experiment on 420 g. of larvae after experience had been gained on other samples.

Fraction 1. This was eluted by the least polar solvent mixture, and showed only an inflexion in the region 260–300 m μ and a weak maximum near 232 m μ (Fig. 1). The material (about 0.3 g.) was dissolved in hot ethanol and colourless crystals were deposited on cooling. The recrystallized material (m.p. 59.5–60°; C, 85.3; H, 14.3%) appeared to be a hydrocarbon. Infrared spectroscopy showed peaks at 3.43, 3.53, 3.78, 6.79, 6.82, 7.26, 11.28, 13.73 and 13.92 μ , consistent with a saturated hydrocarbon. Because the bands at 6.82 μ (C–H deformation of CH₂ groups) and 7.26 μ (C–H deformation of CH₃ groups) are of the same intensity, the hydrocarbon probably has a branched-chain structure.

A preliminary mass-spectroscopic study carried out by Dr R. I. Reid and Dr J. Wilson, Department of Chemistry, University of Glasgow, has shown the hydrocarbon to have a molecular weight of 630 ± 2 , consistent with the formula C₄₅H₉₂.

Fraction 2. This showed a peak at 272 m μ (Fig. 1) and the absorption curve was typical of ubiquinone. Reversed-phase chromatography on paper of 0.1 mg. of this fraction showed that it contained three ubiquinones, namely those with side chains containing 40, 45 and 50 carbon atoms respectively. A larger sample was separated as described and the isoprenologues were extracted from sections of paper and assayed spectrophotometrically. The results indicated the presence of ubiquinones-40, -45 and -50 in the proportion 2:10:7 respectively.

Fig. 1. Absorption curves of chromatographic fractions from unsaponifiable matter of blowfly larvae. Fraction 1, (---) in cyclohexane (1 cm., 0.0303%); fraction 2, (—) in cyclohexane (1 cm., 0.0063%); fraction 3, (— · —) in cyclohexane (1 cm., 0.0323%).

Fraction 3. This showed only ill-defined selective absorption, possibly due to a trace of ubiquinone. The material (about 0.03 g.) was acetylated (acetic anhydride, pyridine) and the product was rechromatographed on alumina. A substantial proportion of the material was readily eluted by means of light petroleum, indicating that the fraction contained much saturated (aliphatic) alcohol (possibly 20 mg.). The later fractions of acetylated product gave no indications of the presence of ubiquinone (Laidman *et al.* 1960) or of dolichol (Pennock, Hemming & Morton, 1960; Hemming, Morton & Pennock, 1960) in more than trace amounts.

Fraction 4. This consisted almost entirely of sterol. The ultraviolet absorption was clearly due to 7-dehydro steroid but, from its intensity, 92–93% of the weight is due to transparent sterol. On this basis the larvae contained about 800 $\mu\text{g.}$ of steroid/g., about 6 $\mu\text{g./g.}$ being 7-dehydro steroid.

Fraction 5. This fraction, eluted by ether, was small in weight and, with a main peak at 296 $\text{m}\mu$ (Fig. 2), may possibly have been due to a steroid with a conjugated-triene chromophoric group (Dorfman, 1953).

Fraction 6. This fraction, eluted by methylal, showed no identifiable selective absorption and was small in weight. The recovery from the chromatography (about 67.5%) was moderately good. The nature of the retained material has not yet been established.

In another experiment 2.23 kg. of larvae was used and exposure to the disintegrating action of the blender was quite brief. After saponification many exoskeletons remained and could be separated, washed, dried and weighed (6% of the initial weight of larvae). The larger amount of unsaponifiable matter obtained in this experiment permitted a more thorough search for ubiquinone. The ultraviolet absorption of the post-ubiquinone fraction showed no evidence of the presence of ubiquinone and rechromatography on magnesia failed to bring to light even traces. Further, the sterol-rich fraction which might have contained ubiquinone was treated with digitonin to remove precipitable 3β -hydroxy sterols; after filtration, the residue of the fraction was free from ubiquinone. The only identifiable substance present was lanosterol which was crystallized in small amounts and showed the expected infrared absorption, Liebermann–Burchard reaction and mixed melting point.

A portion of fraction 2 was acetylated and rechromatographed on alumina. By this means α -tocopheryl acetate can be separated from ubiquinone. None, however, was found.

RESULTS AND DISCUSSION

Table 2 records the findings of this study.

Cain & Morton (1955) have discussed the occurrence of hydrocarbons in a number of mammalian tissues. They mention a number of saturated hydrocarbons ranging in size from C_{25} to C_{30} . Although the compound obtained from blowfly larvae had a melting point (59–60°) near to those of the mammalian hydrocarbons, mass spectra showed that it was clearly a larger molecule. The isolation procedure used to obtain the hydrocarbon from blowfly larvae involved simple adsorption chromatography followed by crystalliza-

tion of the hydrocarbon fraction from one solvent only. It is, therefore, quite possible that the product may have been a mixture of related hydrocarbons. This possibility does not, however, detract from the proof, by means of the mass spectrometer, that a large hydrocarbon ($\text{C}_{45}\text{H}_{92}$, mol.wt. 630) was present in the preparation.

Chibnall, Piper, Pollard, Williams & Sahai (1934) recorded the occurrence of an homologous series of saturated hydrocarbons (odd numbers from C_{25} to C_{35}) and of a series of primary alcohols (even

Fig. 2. Absorption curves of chromatographic fraction from unsaponifiable matter of blowfly larvae. Fraction 4, (---) in cyclohexane (1 cm., 0.0338%); fraction 5, (—) in cyclohexane (1 cm., 0.0115%); fraction 6, (— · —) in cyclohexane (1 cm. 0.0094%).

Table 2. *Estimated amounts of minor constituents of unsaponifiable matter obtained from blowfly larvae*

Amounts are expressed in $\mu\text{g./g.}$ of larva (fresh wt.). N.D., Not detected.

Saturated hydrocarbon	100
Unsaturated hydrocarbon	N.D.
α -Tocopherol	N.D.
Ubiquinone-40	5
Ubiquinone-45	24
Ubiquinone-50	17
Ubichromenol	N.D.
Dolichol	N.D.
7-Dehydro sterol	6
Lanosterol	6
Total sterol	810
α -Tocopherol quinone	N.D.
Vitamin A	N.D.

numbers from C_{24} to C_{36}) in insect waxes. The saturated-hydrocarbon fraction isolated from the larvae of the hide beetle *Dermestes vulpinus* by Clark & Bloch (1959a) was probably such a mixture. The hide-beetle larvae were able to synthesize the hydrocarbon from ^{14}C -labelled acetate and the results of C -methyl analysis of the radioactive hydrocarbon indicated that it had an unbranched structure.

Elucidation of the function of a hydrocarbon such as that found in blowfly larvae demands among other things a knowledge of the location of the material within the larvae and within the larval cell. This remains to be done.

The alcohol fraction from the chromatography of the unsaponifiable lipids from *Dermestes vulpinus* had an infrared spectrum which the authors associated with a straight-chain saturated alcohol or a mixture of such alcohols. Acetylation of their preparation with ^{14}C -labelled acetic anhydride and determination of the degree of isotope dilution in the resultant acetate lead to a value of 437 ± 17 for the average molecular weight of the mixture of alcohols. This value is consistent with a C_{25} or C_{26} saturated alcohol. Blowfly larvae obviously contained similar alcohols.

The discovery of three ubiquinone isoprenologues in blowfly larvae is unexpected; other investigations of insect tissues have revealed only one isoprenologue. Lester & Crane (1959) found that coenzyme Q_9 (ubiquinone-45) was present in the housefly *Musca domestica* and in the cabbage-white butterfly *Pieris rapae*. On the other hand, Heller, Szarkowska & Michalek (1960) found ubiquinone-50 in the hawk moth *Celeris euphorbiae*. They also examined the pupae of *Sphinx pinastri* (Lepidoptera) and the adult insects of *Anatis ocellata* (Coleoptera). These too contained ubiquinone-50. The multiplicity of ubiquinone isoprenologues in the blowfly larvae may reflect the nature of the ubiquinone content of the larval diet. Further experimentation will be necessary to establish such a relationship, but a number of studies on insect metabolism lend weight to such a hypothesis. Insects are unable to synthesize their own sterols (Lipke & Fraenkel, 1956; Clark & Bloch, 1959b), and Wootton & Wright (1960) have shown that the earthworm *Lumbricus terrestris*, with a nearly sterile gut after being on a penicillin-treated medium, is unable to synthesize either sterols or ubiquinone from $[^{14}C]$ mevalonic acid injected into the coelomic cavity. Invertebrates, with a few exceptions, are unable to convert β -carotene into vitamin A (Moore, 1957). It may be that insects and invertebrates in general are unable to synthesize polyisoprenoid compounds, so that the presence or absence of the various members of this group of compounds in any invertebrate

species will depend on the diet and intestinal absorptive powers of that species. If invertebrates are unable to synthesize sterols, the biosynthetic origin of the somewhat random mixtures of phyto-sterols and zoosterols found in many invertebrates by Bock & Wetter (1938) is doubtful. The sterol fraction from earthworm, for example, was found to contain ergosterol, cholesterol and stigmasterol.

SUMMARY

1. Saponification of the larvae of the blowfly *Calliphora erythrocephala* in the presence of pyrogallol yielded 0.27% of unsaponifiable matter.

2. Adsorption chromatography on alumina allowed a number of constituents of the unsaponifiable matter from blowfly larvae to be isolated and identified.

3. Blowfly larvae were found to contain 5 μ g. of ubiquinone-40/g., 24 μ g. of ubiquinone-45/g. and 17 μ g. of ubiquinone-50/g. The larvae also contained 6 μ g. of lanosterol/g., 6 μ g. of 7-dehydrosterol/g. and 100 μ g. of saturated hydrocarbon of undetermined structure/g.

4. Unsaturated hydrocarbons, α -tocopherol, ubiquinone, dolichol, α -tocopherol quinone and vitamin A were not found in blowfly larvae.

5. Some aspects of lipid biochemistry in insects have been discussed.

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