

Dolichol: a Naturally-Occurring C₁₀₀ Isoprenoid Alcohol

By J. BURGOS, F. W. HEMMING, J. F. PENNOCK AND R. A. MORTON

Department of Biochemistry, The University of Liverpool

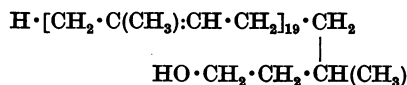
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The unsaponifiable fractions from the lipids of plant and animal tissues contain a number of polyisoprenoid compounds. Squalene and one or other of the ubiquinones occur very often. Occasionally vitamin K₂ is isolated from animal tissues, and green plant tissues contain relatively large amounts of plastoquinone and much smaller amounts of vitamin K₁. A series of tocotrienols, in which the side chain of the tocol nucleus is isoprenoid, have been isolated from seed oils (Bunyan, McHale, Green & Marcinkiewicz, 1961).

It is well established that squalene is formed enzymically by the condensation of two molecules of farnesyl pyrophosphate, with the elimination of phosphate. The work of Martius's group (Stoffel & Martius, 1960) indicates that a mitochondrial enzyme catalyses the condensation of solanesol pyrophosphate with the appropriate quinone to form ubiquinone-45 or vitamin K₂-45. Solanesol itself has been isolated from both green and cured tobacco leaves (Rowland, Latimer & Giles, 1956). In the green leaf its biosynthesis from mevalonate has been reported (Reid, 1961), but the origin of solanesol in animal tissues has not yet been established.

These advances stimulated a search in many tissues for long-chain isoprenoid alcohols and although plant tissues yielded a number of compounds of this type (Pennock, Hemming & Morton, 1963; J. Stevenson, unpublished work), animal tissues, and in particular human kidney and pig liver, have yielded only one such alcohol. This had an unusually large molecular weight and it was called dolichol (Greek *dolichos*, long). Dolichol has been found to be present in human kidney and liver, pig pancreas, spleen and liver, sheep brain, ox intestine, in the liver, kidney, intestine and skeletal muscle of the rabbit and in the spadix of *Arum maculatum*. Baker's yeast also contains an, as yet, incompletely characterized alcohol (3 mg./kg. wet wt.) that closely resembles dolichol but is more polar when subjected to paper chromatography. Pennock, Hemming & Morton (1960) described briefly the isolation and properties of dolichol from human kidney. A further sample of dolichol was isolated from pig liver and the two compounds had identical infrared spectra and could not be separated by paper chromatography. In the present

paper the properties of the compound obtained from both sources are described fully. Evidence is presented and discussed for the structure:



15 or 16 of the internal isoprenoid residues are in the *cis* configuration.

EXPERIMENTAL

Isolation of dolichol. (a) From pig liver. We are indebted to Hoffmann-La Roche and Co., Basle, in particular to Dr U. Gloor, who supplied us with 106 g. of unsaponifiable lipid obtained from ten batches of pig liver. Each batch (10 kg.) had been digested by refluxing with a mixture of 3 kg. of KOH, 2 l. of water, 100 g. of pyrogallol and 2 l. of ethanol for 2½ hr. under N₂. The solution was then extracted three times with ether and the extract washed with water, dried and the solvent distilled off. The ten batches yielded a total of 278 g. of unsaponifiable material and this was dissolved in 2.78 l. of light petroleum (b.p. 60–90°) and cooled to 3°. The sterols were filtered off, washed three times with cold light petroleum and the combined filtrates, on removal of the solvent *in vacuo*, yielded 106 g. of material.

This was then dissolved in petrol (light petroleum; b.p. 40–60°) and the solution was divided into four portions. One portion was mixed with a slurry of Brockmann grade 3 alumina (1 kg.) in petrol (1 l.) in a 3 l. Erlenmeyer flask. After shaking, the alumina was allowed to settle and the supernatant decanted off. Washing the alumina by shaking with a further 3 × 1.6 l. of petrol and 3 × 1.6 l. of 2% ether in petrol, and decanting each time, removed 2.9 g. of material. Washing further with 4 × 1.6 l. of 15% ether in petrol followed by 4 × 1.6 l. of 30% ether in petrol yielded a total of 5.8 g. of lipid, which was chromatographed on 250 g. of Brockmann grade 3 alumina in a column of 6 cm. diameter. Elution of the column with 3 × 250 ml. of 4% ether in petrol removed 786 mg. of material, which did not contain dolichol since it showed no hydroxyl band in the infrared spectrum. A fourth portion of 4% ether in petrol (250 ml.) eluted dolichol-containing material (bands in the infrared at 2.95, 6.00, 11.26 and 11.97 μ) as did 250 ml. of 7% ether in petrol and 250 ml. of 10% ether in petrol. When bulked, these three fractions yielded 3.6 g. of lipid, which was then acetylated by dissolving in a mixture of benzene (30 ml.) and pyridine (15 ml.), adding acetic anhydride (25 ml.) and leaving overnight at room temperature. The acetate was extracted and chromatographed on alumina in a way similar to that described below for the *p*-phenylazobenzoate.

The other three portions of the 'sterol-free' unsaponi-

fiable lipid were treated in essentially the same way and a total (from all four portions) of 11.05 g. of acetates was obtained. The acetates were saponified and the almost pure alcohol was then crystallized six times from petrol-ethanol mixtures at -20° . The last two crystallizations brought about no change in infrared absorption of a molten film and the material ran as a single entity on a paper chromatogram (R_f 0.45 on paper impregnated with paraffin, mobile phase acetone). In this way 6 g. of pure dolichol was obtained.

(b) From human kidney. The isolation of ubiquinone from human kidney (46.5 kg.) has already been described in detail (Laidman, Morton, Paterson & Pennock, 1960). The tissue (46.5 kg.) was saponified to yield unsaponifiable lipid (140.75 g.), which was concentrated, by crystallizing the sterols from solution in petrol, to a weight of 41.2 g. Chromatography on columns of alumina yielded fractions of ubiquinone (eluted by 5% ether in petrol) and ubiquinone (eluted by 10% ether in petrol). During purification of the ubiquinone on alumina and MgO two fractions (5.01 and 1.40 g.) were removed as impurities (for details see Laidman *et al.* 1960). These were bulked and the infrared spectrum indicated the presence of an isoprenoid alcohol which we now call dolichol. After preparation in a way similar to that described for pig-liver dolichol, 2 g. of pure material was obtained.

Dolichol p-phenylazobenzoate and p-nitrobenzoate. These esters were formed by refluxing for 3 hr. a three- to five-fold excess of the corresponding acid chloride with dolichol dissolved in toluene (50 vol.) containing a few drops of pyridine. The solution was then poured into ice-cold 2N-HCl and the mixture was extracted three times with ether. The ethereal solution was washed free from acid and, on removal of the solvent, the ester was purified by chromatography on acid-washed Brockmann grade 3 alumina. A loading of 50 mg. on 10 g. of alumina in a column of 1.4 cm. diameter was used. After passing 100 ml. of petrol through the column the ester was eluted by 100 ml. of 1% ether in petrol followed by 100 ml. of 2% ether in petrol. Further purification was by crystallization at -20° from solution in petrol-ethanol mixtures.

Spectra. Ultraviolet absorption at wavelengths above 220μ was measured in a Unicam SP. 500 spectrophotometer. The absorption of dolichol and solanesol in the region 190–210 μ was determined by Dr C. von Planta (Hoffmann-La Roche and Co., Basle) using a vacuum grating spectrometer as described by Planta (1962).

Infrared-absorption spectra were recorded by a Perkin-Elmer Infracord (model 137) spectrometer. The high-resolution spectra were obtained by Dr C. H. Steele of the Thornton Research Laboratories, Shell, Ellesmere Port, Cheshire, with a Grubb-Parsons double-beam grating spectrometer (model GS2). Samples were examined as films between NaCl plates whenever possible. Materials of high melting point were studied as KBr disks.

Nuclear-magnetic-resonance spectra were obtained at 60 Mcyc./sec. on a Varian A 60 instrument by Dr R. J. Abraham and Mr J. V. Barkley of the Department of Organic Chemistry, The University of Liverpool. Compounds were examined as solutions in carbon tetrachloride. Tetramethylsilane was used as reference substance.

Mass spectra were determined by Dr R. I. Reed of the Department of Chemistry, The University of Glasgow.

Quantitative catalytic hydrogenation of the alcohols. This

was carried out in a Towers microhydrogenation apparatus with Adams catalyst and ethanol-cyclohexane-acetic acid (1:1:1, by vol.) as solvent.

Citronellol. This was formed by reducing citronellal (5 g.) (kindly given by Hoffmann-La Roche and Co., Basle), dissolved in methanol (25 ml.) with excess of sodium borohydride. When effervescence had ceased the solution was poured into ether (250 ml.) and the mixture washed with water (5×200 ml.). The ether was evaporated and the infrared absorption of the product (5 g.) indicated that all the citronellal had been reduced to citronellol.

Ozonolysis of citronellol p-nitrobenzoate. Citronellol p-nitrobenzoate was formed by refluxing for 3 hr. a solution of citronellol (1 g.) and p-nitrobenzoyl chloride (2 g.) in benzene (50 ml.) containing a few drops of pyridine. The ester was extracted and chromatographed on alumina as described above for dolichol p-nitrobenzoate, to yield citronellol p-nitrobenzoate (2.1 g.). Of this, 1.02 g. was dissolved in ethyl acetate (20 ml.) and the solution was ozonized for 30 min. at 0° . Excess of ozone was removed by bubbling N_2 through the solution and then H_2O_2 (10 vol.; 20 ml.) was added. This addition of H_2O_2 was repeated after 1 hr. and again after a further hour had elapsed. Extraction with ether and evaporation of the solvent yielded a pale-yellow oil (824 mg.), a portion of which (350 mg.) was chromatographed on a column of silicic acid (Mallinckrodt, 50 g.). Benzene (50 ml.) eluted a little material but the following fraction eluted by 10% ether in benzene contained a wax (307 mg.). A portion (24 mg.) of this was purified by chromatography on thin layers of silica gel with 75% ether in benzene as solvent. The product was crystallized several times from petrol-ethanol mixtures at -20° . The pale-yellow waxy crystals melted just above room temperature and they were almost insoluble in cyclohexane. For this reason the ultraviolet absorption had to be studied in cyclohexane-ethanol (99:1, v/v).

Ozonolysis of dolichol p-nitrobenzoate. This was carried out on two samples of the ester formed from pig-liver dolichol. The first sample (194.3 mg.) was dissolved in 10 ml. of ethyl acetate and the methods of ozonolysis, degradation of the ozonide and purification of the product showing ultraviolet absorption were similar to those described for citronellol p-nitrobenzoate.

A second sample (650 mg.) of dolichol p-nitrobenzoate was dissolved in 100 ml. of ethyl acetate and ozonized as before. The ozonide was split reductively by adding zinc dust (about 200 mg.) and a drop of acetic acid. The 2,4-dinitrophenylhydrazones of the products were then formed and studied.

RESULTS AND DISCUSSION

Dolichol is an odourless, colourless, viscous oil at room temperature. It melts at slightly above -10° .

Presence of a hydroxyl group. The infrared spectrum of dolichol (Fig. 1) has a band of medium strength at 2.95μ , which implies the presence of a hydroxyl group in the molecule. This was confirmed when it was found that the molecule could be acetylated; the acetate no longer absorbed at 2.95μ but had strong absorption bands at 5.72 and 8.12μ as expected (see Fig. 2).

Equivalent weight and molecular weight of the alcohol. The formation of an ester was used as a means of determining the equivalent weight of the alcohol. A sample of dolichol was sent to Hoffmann-La Roche and Co., Basle, and we are indebted to Dr O. Isler, Dr U. Gloor and Dr J. Würsch, who formed the acetate by using ^{14}C -labelled acetic anhydride according to the method of Kofler *et al.* (1959). The acetate was carefully purified, finally on thin layers of silica gel, and the dilution of the isotope corresponded with equivalent weight 1419 for the acetate. The equivalent weight of dolichol is therefore about 1380. We had ourselves used a method involving the ultraviolet absorption of esters.

The chromophores of the acid radicals *p*-phenylazobenzoate and *p*-nitrobenzoate exhibit characteristic absorption in the ultraviolet region of the spectrum (see Table 1). The corresponding esters of dolichol were prepared and purified by chromatography on alumina followed by crystallization. $E_{1\text{cm}}^{1\%}$ values at maximal absorption were then compared with the mean values for esters of known constitution so that an equivalent weight (or minimal molecular weight) of the alcohol could be calculated. The ultraviolet absorption of the *p*-phenylazobenzoates gave a mean molecular extinction of 26 770; since the mean $E_{1\text{cm}}^{1\%}$ of dolichol *p*-phenylazobenzoate was 171.4, the equivalent weight of the ester was calculated to be 1561. This corresponds with an equivalent weight 1352 for dolichol. In the same way it was calculated that the ultraviolet absorption of the *p*-nitrobenzoates indicates an equivalent weight 1300 for free dolichol. The former is within 2.1% and the latter within 5.8% of the more trustworthy answer obtained by isotope dilution.

There is no evidence that the dolichol molecule has more than one hydroxyl group, and other independent, physical methods indicate that the equivalent weight is also the molecular weight of the alcohol.

A sample of human-kidney dolichol was examined in a mass spectrometer and the largest ion detected contained 100 carbon atoms. If it is assumed that

this was derived without fragmentation of the carbon skeleton or without capture of fragments, the intact dolichol molecule must contain 100 carbon atoms. Other evidence indicates that dolichol is polyisoprenoid, and so this number of carbon atoms fits a molecular weight near 1380.

A molecular-weight determination by depression of the freezing point of cyclohexane gave a value of near 1200, which is in moderately good agreement with the equivalent weight determined as already described.

There is thus little doubt that the molecular weight of dolichol is near 1380. In fact the structure proposed for dolichol corresponds with a molecular weight of 1382.4 and this figure has been used in calculations throughout the rest of this paper.

Analysis. Analysis of human-kidney dolichol (in the Department of Organic Chemistry of The University of Liverpool) gave C 86.07, H 12.18 and also C-Me 16.64%. The proposed structure required C 86.87, H 11.96 and C-Me 22.11%. Kuhn-Roth determinations for C-Me groups are well known to give low values. Figures ranging from 60 to 90% of the theoretical figure for this type of compound have been quoted by Kuhn & Roth (1953) and Campbell & Morton (1952).

Infrared absorption of dolichol, perhydrodolichol and dolichol. The infrared spectra of dolichol and the isoprenoid alcohol solanesol have a number of similarities (see Fig. 1). (Unless otherwise indicated, absorption bands have been assigned to the appropriate groups; Bellamy, 1958.) Both compounds show O-H stretching absorption at 2.95 μ and have absorption at 6.00 μ (unconjugated C:C stretching). The band at 11.97 μ is consistent with the double bonds' being trisubstituted and the intensity of the band at 7.29 μ (C-CH₃ symmetrical deformation) confirms the presence of a large number of methyl groups in the molecule. The infrared evidence is therefore in accord with dolichol's being a polyisoprenoid alcohol.

There are, however, some important differences between the two spectra. The intensity of the hydroxyl absorption band relative to the rest of the

Table 1. *Ultraviolet absorption of esters in cyclohexane*

Alcohol	$E_{1\text{cm}}^{1\%}$	λ_{max}	Equiv. wt.	ϵ	λ_{max}
	<i>p</i> -Phenylazobenzoates (λ_{max} 324 m μ)				
Solanesol	333.0		839.3	27 700	} 26 770
Cholesterol	438.0		594.9	26 060	
Farnesol	616.6		430.6	26 550	
Pig-liver dolichol	171.5	} 171.4	1 561*		
Human-kidney dolichol	171.3				
	<i>p</i> -Nitrobenzoates (λ_{max} 256 m μ)				
Farnesol	379.5		371.5	14 090	} 13 805
Phytol	303.5		445.6	13 520	
Human-kidney dolichol	195.0		1 453*		

* Calc. from ϵ .

spectrum suggests that dolichol is a much larger molecule than solanesol. The band at $10.02\ \mu$ in the solanesol spectrum is due to O-H deformation absorption (Rowland *et al.* 1956), but this band is absent from the spectrum of dolichol. When dolichol was oxidized to the aldehyde, dolichal, a band at $9.49\ \mu$ disappeared (Fig. 2). The perhydro derivatives of solanesol and dolichol gave bands in the infrared at $9.49\ \mu$ (Fig. 3). The band at $10.02\ \mu$ in the spectrum of solanesol is consistent with an allylic primary alcohol ($>C:CH\cdot CH_2\cdot OH$), and therefore a band at $9.49\ \mu$ indicates a simple primary alcohol ($-CH_2\cdot CH_2\cdot OH$) (see also Bellamy, 1958, p. 95).

When the minor bands in the infrared spectra of dolichol and solanesol are considered other differences are noticed (Table 2). If the positions of these bands are compared with those shown by *Hevea* rubber, in which 97.8% of the isoprene units are in the *cis* configuration, and balata rubber, in

which 98.7% of the isoprene units are in the *trans* configuration (Golub, 1959), there is excellent support for the argument that solanesol has only *trans* isoprene units and dolichol has mainly *cis* isoprene units. A comparison with the spectra of all-*trans*-squalene and isomerized squalene containing approximately half of its six isoprene units in the *cis* configuration (Cunneen, Higgins & Watson, 1959) confirms this. As seen below, similar results were obtained from nuclear-magnetic-resonance studies.

Since the infrared-absorption spectrum of perhydrodolichol is qualitatively identical with that of perhydrosolanesol (Fig. 3) it is reasonable to conclude that dolichol can differ from solanesol only (a) in the extent of unsaturation, (b) in the configuration of the double bonds and (c) in the size of the molecule.

High resolution of the C-H stretching absorption in the region near $3.4\ \mu$ revealed other differences between the spectra of solanesol and dolichol. Each has three peaks in this region at approximately the same wavelengths respectively, but the relative intensities of the peaks are different in the two spectra (Fig. 4). It will also be seen (Fig. 5) that in the spectra of the perhydro derivatives, although the triplets are of similar shape, the relative intensity of the central peak is greater in the perhydrosolanesol spectrum than in the perhydrodolichol spectrum. These differences are not fully understood.

Dolichal and farnesal. Both dolichal and farnesal were prepared by refluxing the alcohols in petrol with excess of manganese dioxide for 2 and 1 hr. respectively. The infrared spectrum of dolichal is reproduced in Fig. 2. The position of the C:O stretching band ($5.78\ \mu$) is consistent with the absence of a double bond in the position $\alpha\beta$ to the

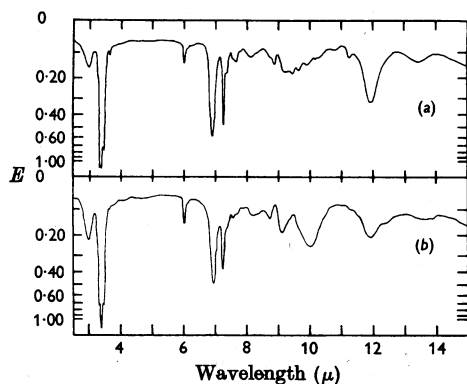


Fig. 1. Infrared-absorption spectra of films of (a) dolichol and (b) solanesol. The films differed in thickness.

Table 2. Infrared absorption of *cis* and *trans* isomers of polyisoprenoid compounds

The data given are for the region $7.5\text{--}11.5\ \mu$ but no reference is made to the O-H absorption. The columns headed 'Balata rubber', 'Isomerized *Hevea* rubber' and '*Hevea* rubber' are from Golub (1959). The figures for isomerized squalene are from Cunneen *et al.* (1959). 'Masked' refers to masking by O-H deformation absorption. ~, Inflection; w, weak; b, broad; n.r., not recorded.

Solanesol all- <i>trans</i>	Balata rubber 98.7% <i>trans</i>	Squalene all- <i>trans</i>	Isomerized squalene about 50% <i>trans</i>	Isomerized <i>Hevea</i> rubber about 50% <i>cis</i>	Dolichol	<i>Hevea</i> rubber 97.8% <i>cis</i>
7.53	7.52	7.53	7.52 w	7.55	7.54 ~	
		7.65 ~	7.63	7.65		7.68
8.10 ~	n.r.	7.97 ~	8.00	n.r.	8.05	n.r.
8.19		8.15			8.19 ~	
8.71	8.70	8.70	8.70 w	8.70		
			8.85	8.80 b	8.88	8.88
9.10	9.10	9.10	9.17	9.18 b	9.21	9.25
Masked	9.70	9.70 b	9.52	9.67	9.63	9.65
Masked	n.r.		9.90	n.r.	9.91	n.r.
11.23 ~	11.30 ~		n.r.	11.30	11.26	11.30

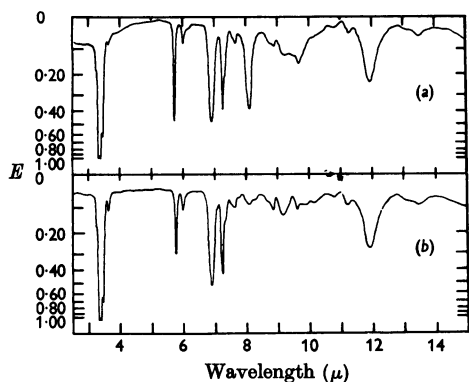


Fig. 2. Infrared-absorption spectra of films of (a) dolichol acetate and (b) dolichal. The films differed in thickness.

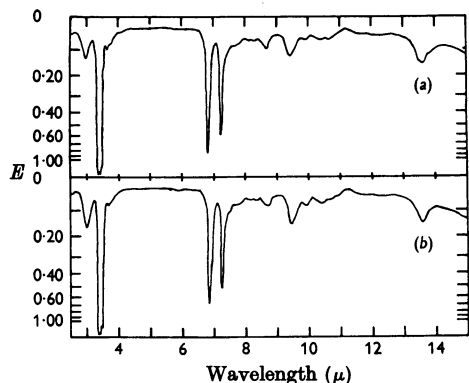


Fig. 3. Infrared-absorption spectra of films of (a) perhydrodolichol and (b) perhydrosolanesol. The films differed in thickness.

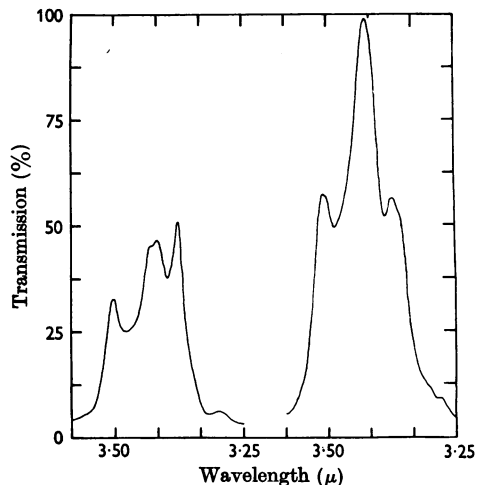


Fig. 4. High-resolution infrared-absorption spectra of films of dolichol (left) and solanesol (right). The films differed in thickness.

aldehyde group. Farnesal had its C:O stretching absorption at 5.97μ and this, together with a shift of the C:C band to longer wavelengths than the normal 6.00μ , is the absorption expected of an $\alpha\beta$ -unsaturated aldehyde.

The 2,4-dinitrophenylhydrazones of these aldehydes were prepared and their ultraviolet-absorption spectra were studied. The spectrum of the dolichal derivative had a peak at $360 m\mu$ with $E_{1\%}^{1\text{cm}}$ 156.1. That of farnesal had a peak at $383 m\mu$ ($E_{1\%}^{1\text{cm}}$ 743.9). The wavelengths of these absorption bands confirm the presence of an $\alpha\beta$ double bond in farnesal and its absence in dolichal, and their intensity reflects the large size of the dolichol molecule (see also Gillam & Stern, 1954).

This work with dolichal strengthens the case that dolichol is a long-chain primary alcohol with the end grouping $-\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}$.

Nuclear-magnetic-resonance spectra of dolichol, solanesol and the perhydro derivatives. The spectra of dolichol, solanesol and perhydrodolichol are reproduced in Figs. 6–8 respectively. The positions of the bands and their relative areas are recorded for dolichol in Table 3, for solanesol in Table 4 and for perhydrodolichol in Table 5.

In assigning the bands of the spectra a number of publications (Jackman, 1959; Tiers, 1958; Chamberlain, 1959) were consulted and the important points were noted as follows. In the dolichol spectrum the strong bands at 8.4 and 8.0τ fit the

methyl protons of $-\text{C}\cdot\text{CH}_3$ and the methylene pro-

tons of $-\overset{\text{H}}{\text{C}}\cdot\text{C}_2-$ plus $-\overset{\text{H}}{\text{C}}\cdot\text{CH}_2-$. A closer inspection of the 8.0τ band and of the literature indicates that

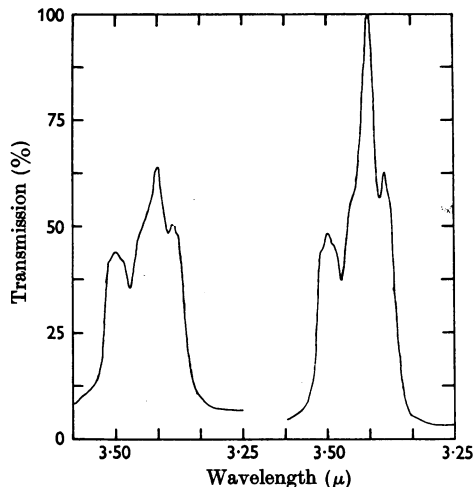


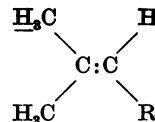
Fig. 5. High-resolution infrared-absorption spectra of films of perhydrodolichol (left) and perhydrosolanesol (right). The films differed in thickness.

the splitting to display bands at 8.02 τ and 7.97 τ

reflects the ability to distinguish between $\begin{array}{c} \text{CH}_3 \\ | \\ =\text{C}\cdot\text{CH}_2 \end{array}$

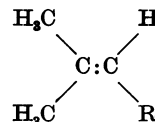
$\begin{array}{c} \text{H} \\ | \\ =\text{C}\cdot\text{CH}_2 \end{array}$ (8.02 τ) and $\begin{array}{c} \text{H} \\ | \\ =\text{C}\cdot\text{CH}_2 \end{array}$ (7.97 τ) (see Tiers, 1958, reference to squalene). The splitting of the 8.4 τ band indicates a differentiation of the *cis* from *trans* substitution of olefinic groups. The band at 8.34 is due to protons of methyl groups *cis* to olefinic protons and that at 8.42 is due to methyl groups *trans* to olefinic protons (Bates & Gale, 1960). When examined in benzene the separation of these two peaks was increased to 0.13 τ (cf. Chen, 1962) and the areas under the peaks could be compared more

accurately. Such measurements indicated the presence of three or four *trans* isoprene units for every 19 isoprene units present. Since one of these must be a terminal methyl group i.e. one of



it follows that two or three of the internal isoprene units are in the *trans* configuration and the other 16 or 15 in the *cis* configuration.

The solanesol spectrum shows a similar splitting at 8.0 τ , but at 8.4 τ the strength of the two peaks is reversed. This is consistent with solanesol's being all-*trans*. The area under the band at 8.34 τ is about one-eighth of that at 8.42 τ and is consistent with only one methyl group i.e. a terminal methyl of



being *cis* to the olefinic proton.

The hydroxyl proton band was detected in the nuclear-magnetic-resonance spectra of solanesol and dolichol by recording them before and after shaking with deuterium oxide. The amplified traces show the effect in each case, the uppermost tracing being that after treatment (Figs. 6, 7 and 8). With dolichol, deuterium oxide eliminated the band at 7.70 τ , which had an area equivalent to one proton. This was therefore assumed to be the band due to the hydroxyl proton. In the same way the absorption band of the hydroxyl proton in solanesol was shown to be that at 8.78 τ . It is well known that

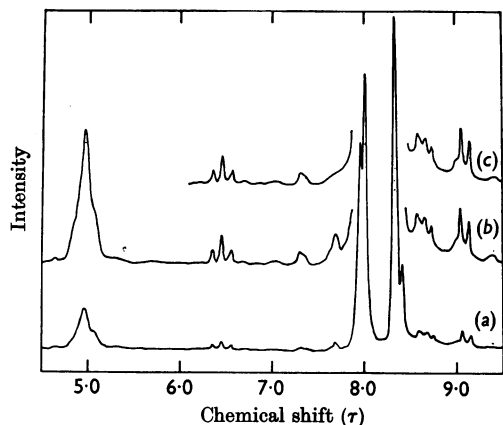


Fig. 6. (a) Nuclear-magnetic-resonance spectrum of pig-liver dolichol in carbon tetrachloride at 60 Mc/sec.; (b) the same amplified; (c) the same after shaking with deuterium oxide, and amplified.

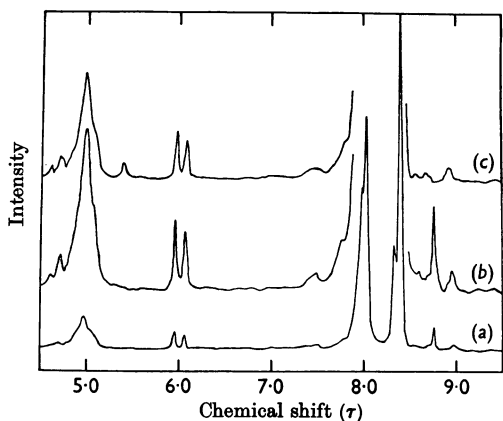


Fig. 7. (a) Nuclear-magnetic-resonance spectrum of solanesol in carbon tetrachloride at 60 Mc/sec.; (b) the same amplified; (c) the same after shaking with deuterium oxide, and amplified.

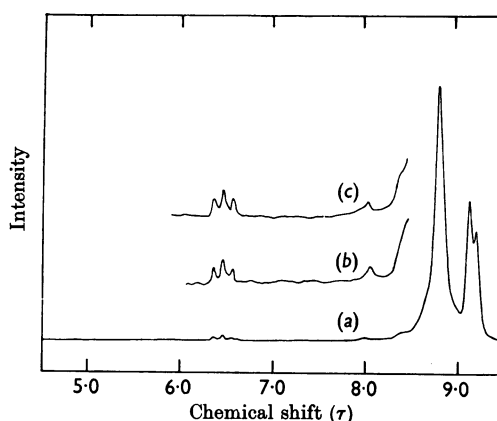


Fig. 8. (a) Nuclear-magnetic-resonance spectrum of per-hydrodolichol in carbon tetrachloride at 60 Mc/sec.; (b) the same amplified; (c) the same after shaking with deuterium oxide, and amplified.

the positions of bands assigned to hydroxyl protons vary considerably (Jackman, 1959).

The protons on the carbon atom next to the hydroxyl group give a band in the region of 6.45 τ in the dolichol spectrum. This is characteristic of the grouping $-\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}$. It was also present in the spectra of perhydrodolichol and perhydro-solanesol and in all three the band was split into a triplet because of the influence of the neighbouring methylene group. In solanesol and farnesol the

double bond $\alpha\beta$ to the hydroxyl group caused a shift of the band to 6.0 τ , where it was split into a doublet since spin-spin interaction occurred with only one neighbouring proton, namely that of $=\text{CH}-$. In the dolichol spectrum the area under the olefinic ($=\text{CH}-$) proton band (4.96 τ) corresponds well with 19 trisubstituted olefinic groups when compared with the area under the $-\text{CH}_2\cdot\text{OH}$ band, which must be equivalent to two protons. The spectrum of dolichol in the 8.5-9.2 τ region supports

Table 3. Nuclear-magnetic-resonance data for pig-liver dolichol

The figures in parentheses in the last column indicate the number of the groups involved in the assignment. In this column the protons are underlined. For the differentiation between the pairs of values in the last column see text.

Positions of bands τ	Relative areas		Assignment
	Found	Expected	
9.15	2.9	3.0	$\text{CH}_3\cdot\text{CH}$
9.05			
8.74	5.6	5.0	CH_3
8.67			
8.58			$-\text{CH}_2\cdot\text{CH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}$
8.42	61.5	60.0	<i>trans</i> (3 or 4)
8.34			<i>cis</i> (17 or 16) $\text{CH}_3\cdot\text{C}:\text{C}\cdot\text{H}$
8.02	71.3	74.0	
7.97			(18) $-\text{CH}_2\cdot\text{C}:\text{C}-$ (19) $-\text{CH}_2\cdot\text{CH}:\text{C}-$
7.70*	1.2	1.0	$\text{O}\cdot\text{H}$
7.31	0.7	—	Impurity?
6.56	1.9	2.0	$-\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}$
6.45			
6.39			
4.96	18.7	19.0	(19) $\text{CH}:\text{C}$
Total	163.8	164.0	—

* Disappeared on shaking with deuterium oxide.

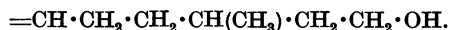
Table 4. Nuclear-magnetic-resonance data for solanesol

For explanation of the last column, see Table 3.

Positions of bands τ	Relative areas		Assignment
	Found	Expected	
8.99	0.2	—	Impurity?
8.78*	1.1	1.0	$\text{O}\cdot\text{H}$
8.42	29.1	30.0	<i>trans</i> (9)
8.34			<i>cis</i> (1) $\text{CH}_3\cdot\text{C}:\text{C}\cdot\text{H}$
8.02	32.2	32.0	(8) $-\text{CH}_2\cdot\text{C}:\text{C}-$
7.98			(8) $-\text{CH}_2\cdot\text{CH}:\text{C}-$
7.48	0.1	—	Impurity?
6.04	2.0	2.0	$=\text{CH}\cdot\text{CH}_2\cdot\text{OH}$
5.96			
4.97	9.0	9.0	(9) $\text{CH}:\text{C}$
4.71			
4.63			
Total	73.7	74.0	—

* Disappeared on shaking with deuterium oxide.

the proposed structure. The doublet at 9.1 τ , both in position and area, fits a methyl group attached to a methine group, i.e. $\text{H}_3\text{C}\cdot\text{CH}$. The absorption in the region 8.58–8.74 τ fits (both qualitatively and quantitatively) the protons of the two methylene and one methine groups underlined in the hydroxyl and of the proposed structure, thus:



The spectrum of perhydrodolichol is that expected from a hydrogenated polyisoprenoid alcohol. The methyl protons are responsible for the band at 9.15 τ ; splitting of the band is due to the presence of the proton on the neighbouring methine groups, namely $-\text{CH}_2\cdot\text{CH}(\text{CH}_3)\cdot\text{CH}_2-$ and $(\text{H}_3\text{C})_2\text{CH}\cdot\text{CH}_2-$. The protons of methylene and methine groups give rise to the absorption at 8.80 τ . The triplet at 6.45 τ due to the protons of the α -methylene group in $-\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}$ is quite clear, but the hydroxyl proton band is not obvious and it may well have moved under the larger peaks. Migration of such bands is well known (Jackman, 1959, p. 66).

The spectrum of perhydrosolanesol is qualitatively identical with that of perhydrodolichol.

Unsaturation. A number of independent methods were used to determine the degree of unsaturation of the molecule. Dr C. von Planta (Hoffmann-La Roche and Co., Basle) studied the ultraviolet

absorption of solanesol and pig-liver dolichol in cyclohexane, in the 190–210 $m\mu$ region. Solanesol had maximal absorption at 196 $m\mu$ with ϵ 8.07×10^4 . Since solanesol has nine double bonds this is equivalent to an extinction coefficient per isoprene unit of 0.90×10^4 . This is the same as that obtained with a different sample of solanesol (Planta, 1962). Dolichol had maximal absorption at 198 $m\mu$, with ϵ 16.25×10^4 . Assuming the extinction coefficient per isoprene unit to be the same in dolichol and solanesol one can calculate that dolichol has 18.1 isoprene units.

The results of hydrogen uptake by pig-liver dolichol and by human-kidney dolichol and its acetate are recorded in Table 6. The hydrogenation apparatus, as used in our Laboratory, has always given values a little higher than expected. For instance, the ethylenic linkages of ubiquinone-50, ubiquinol-50 diacetate and ubichromenol-50 gave hydrogen uptakes of 10.4, 10.3 and 10.7 moles of hydrogen/mole respectively (expected value, 10).

Table 6 also contains the results of iodine uptake by the three compounds, determined according to the method of Dam (1925).

There are some discrepancies between the results based on the various methods, but there is no doubt that the molecule of dolichol contains at least 18 and at most 21 double bonds. Nuclear-magnetic-

Table 5. Nuclear-magnetic-resonance data for perhydrodolichol

Positions of bands τ	Relative areas		Assignment
	Found	Expected	
9.17 9.11	65.0	63.0	(21)+ $\text{CH}_3\cdot\text{CH}$ CH_3
8.80	135.0	136.0	(1)+ $\text{CH}_3\cdot\text{CH}\cdot\text{CH}_2\cdot\text{CH}_2-$ (18)+ $-\text{CH}_2\cdot\text{CH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2-$ CH_3 (1)+ $-\text{CH}_2\cdot\text{CH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}$ CH_3
6.53 6.45 6.39	1.5	2.0	$\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}$
Not located	—	1.0	$\text{O}\cdot\text{H}$
Total	201.5	202.0	—

Table 6. Degree of unsaturation of dolichol

Material	Hydrogen uptake		Iodine uptake	
	moles/100 g.	moles/mole	moles/mole	g./100 g.
Dolichol from pig liver	1.484	20.5	19.4	356.0
Dolichol from human kidney	1.465	20.2	20.8	382.0
Acetate from human-kidney dolichol	1.424	20.3	20.7	369.4

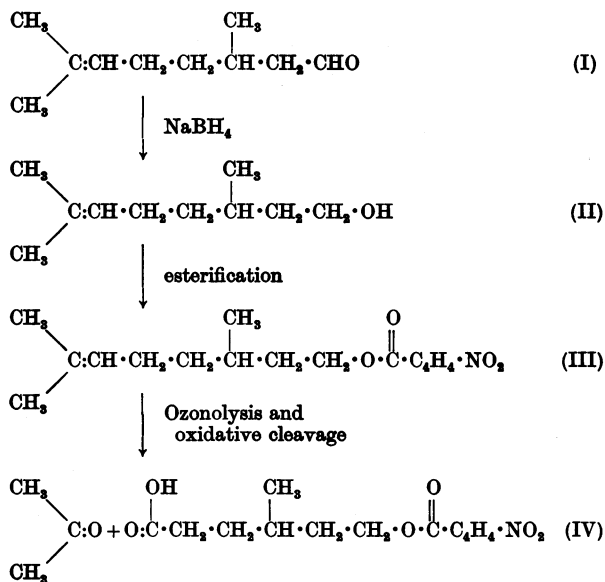


Fig. 9. Preparation of 4-methyl-6-*p*-nitrobenzoylhexanoic acid from citronellal.

resonance studies indicated the presence of 19 double bonds, and this would fit the proposed structure. The results of degradation by ozonolysis (see below) are also consistent with this structure.

Ozonolysis. If the proposed structure for dolichol is correct, then the hydroxyl end of the molecule is identical with the corresponding end of citronellol. Confirmation of this proposal was attempted by degradative ozonolysis.

Citronellal (I) was reduced with methanolic sodium borohydride to give citronellol (II), which was converted into the *p*-nitrobenzoate (III, Fig. 9). After ozonolysis in ice-cold ethyl acetate the ozonide was split oxidatively and the products (IV, Fig. 9) were extracted with ether. The extract was purified by chromatography on silicic acid and most of the material with λ_{max} 256 μm was eluted with 10% ether in benzene. A portion of this material chromatographed as a line on thin layers of silica gel with 75% ether in benzene as mobile phase. When viewed in ultraviolet light a dark band was visible with R_f 0.45. The material was extracted from this dark band and showed λ_{max} at 257 μm with $E_{1\text{cm}}^{1\%}$ 458 (in cyclohexane containing 1% of ethanol). Crystallization did not alter these values. From the ϵ values in Table 1, the ultraviolet absorption of this derivative indicates molecular weight 301. That expected for 4-methyl-6-*p*-nitrobenzoylhexanoic acid is 295.

The product was a wax and melted just above room temperature. An infrared-absorption spectrum of a molten film of the material (see Fig. 10) was consistent with the presence of a carboxylic

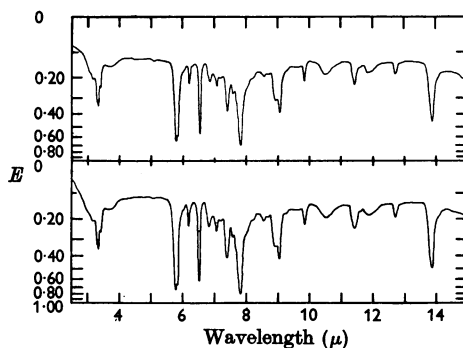


Fig. 10. Infrared-absorption spectrum of a film of the *p*-nitrobenzoate of 4-methyl-6-*p*-nitrobenzoylhexanoic acid derived by ozonolysis of citronellol *p*-nitrobenzoate (upper curve) and of dolichol *p*-nitrobenzoate (lower curve).

acid and a *p*-nitrobenzoate residue (see Randall, Fowler, Fuson & Dangel, 1949).

A small sample (30 μg .) of the product was chromatographed on Whatman 3MM paper, which had been previously soaked with 70% acetic acid and blotted dry. The mobile phase (ascending) was a mixture (9:1) of heptane and 'amyl acetate' (Hopkin and Williams Ltd., Chadwell Heath, Essex) previously saturated with 70% acetic acid; R_f was 0.62.

Dolichol *p*-nitrobenzoate (194.3 mg., prepared from pig-liver dolichol) was treated in the same way as the citronellol *p*-nitrobenzoate. A portion (31.4 mg.) of the products was eluted from the

silicic acid column with 10% ether in benzene, and this was chromatographed on thin layers of silica gel with 75% ether in benzene as solvent. The material absorbing ultraviolet light had R_f 0.45. After extraction from the silica gel, the material was dissolved in cyclohexane containing 1% of ethanol and its ultraviolet-absorption spectrum showed one maximum at 257 $m\mu$ with $E_{1\text{cm}}^{1\%}$ 442.

The infrared spectrum of the material was identical with that of the citronellol *p*-nitrobenzoate derivative (see Fig. 10) and, when chromatographed on the same paper, the two products had identical R_f values (see above).

A second sample (650 mg.) of dolichol *p*-nitrobenzoate was ozonized in ethyl acetate. The ozonide was cleaved reductively with zinc dust and a drop of acetic acid and the mixture was filtered into an excess of a saturated solution of 2,4-dinitrophenylhydrazine in 2*N*-sulphuric acid. The precipitated derivatives were filtered off, washed and combined with those derived from solution in the ethyl acetate.

The mixture was treated in a Soxhlet extractor with petrol and a portion was subjected to descending partition chromatography on paper (Whatman 3MM) impregnated with dimethylformamide. Cyclohexane, saturated with dimethylformamide, was used as the mobile phase and the main band had R_f 0.61, identical with that of acetone 2,4-dinitrophenylhydrazone. Minor bands corresponding to formaldehyde (R_f 0.28), acetaldehyde (R_f 0.42) and butan-2-one (R_f 0.76) 2,4-dinitrophenylhydrazones were also identified but were found to have arisen as impurities from the 2,4-dinitro-

phenylhydrazine. The material with R_f 0.61 was purified further by chromatography on thin layers of silica gel with 75% ether in benzene. The material was crystallized from purified ethanol and the ultraviolet absorption determined. Maxima were found at 225 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 642), 252 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 499), 344 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 965) and 405 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 247), and minima were found at 245 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 480), 287 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 96.5) and 390 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 238).

A comparison of this spectrum with the data in Table 7 shows the similarity in position and intensity of these bands with those of acetone 2,4-dinitrophenylhydrazone. The authentic material and the unknown both melted at 124° (also mixed m.p.). The infrared-absorption spectra of acetone 2,4-dinitrophenylhydrazone and the unknown are identical.

The 2,4-dinitrophenylhydrazone (2.02 g.) remaining in the Soxhlet thimble was dissolved in acetonitrile-toluene (1:1, v/v) and left to crystallize. After two further crystallizations the product had ultraviolet absorption in chloroform with maxima at 258 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 493) and 375 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 904) and minima at 243 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 447) and 295 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 81.2), which was identical with that of the bis-2,4-dinitrophenylhydrazone of laevulinaldehyde (see Table 7). The infrared absorption of the two materials and the R_f values on a thin layer of silica gel (0.48 with 12% ethyl acetate in benzene as solvent) were also the same in each case.

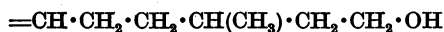
Reductive cleavage of the ozonide of dolichol *p*-nitrobenzoate therefore yielded acetone and laevulinaldehyde; its oxidative cleavage provided 4-methyl-6-*p*-nitrobenzoylhexanoic acid. This is consistent with the proposed structure.

Table 7. Ultraviolet absorption of 2,4-dinitrophenylhydrazones

All the results were obtained in cyclohexane, except those for bislaevulinaldehyde, which were in chloroform. λ_{max} and λ_{min} are recorded in $m\mu$. With the laevulinaldehyde derivative no extinction values below 230 $m\mu$ could be recorded and there was no maximum between 380 and 410 $m\mu$.

	Formaldehyde	Acetaldehyde	Acetone	Bislaevulin- aldehyde	Butan-2-one
λ_{max}	225	225	226		226
$E_{1\text{cm}}^{1\%}$	582	611	642		592
λ_{min}	236	240	245	243	245
$E_{1\text{cm}}^{1\%}$	481	508	480	453	449
λ_{max}	254	253	252	257	252
$E_{1\text{cm}}^{1\%}$	550	550	494	500	458
λ_{min}	283	284	287	294	287
$E_{1\text{cm}}^{1\%}$	139	106	87	90	68
λ_{max}	329.5	338	345	357	345
$E_{1\text{cm}}^{1\%}$	1009	1016	942	911	869
λ_{min}	370	375	390		392
$E_{1\text{cm}}^{1\%}$	263	264	236		197
λ_{max}	383	395	405		407
$E_{1\text{cm}}^{1\%}$	278	282	239		212

Optical activity. Dolichol should be optically active, for the suggested structure contains an asymmetric carbon atom in the groupings

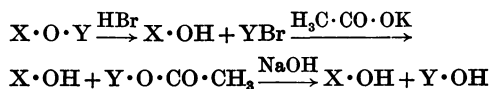


Although the activity in a molecule of this size would be expected to be only slight it was measurable. Dr Burkhardt (Hoffmann-La Roche and Co., Basle) studied the rotatory dispersion at 25° of a solution of pig-liver dolichol in dioxan; it was slightly optically active over the range 360–700 m μ . The accuracy of the results for $[\alpha]$ was $\pm 0.2^\circ$. $[\alpha]$ gradually increased from -0.4° at 700 m μ to -1.8° at 360 m μ .

Thiourea inclusion compound. An attempt was made to form an inclusion compound of dolichol by mixing a saturated solution of thiourea in methanol with a solution of dolichol in benzene, according to the method described by Dicker & Whiting (1958) for squalene. The method was tried first with squalene and a good crop of the characteristic needle-shaped crystals of the inclusion compound was obtained. When dolichol was used, crystals of thiourea and globules of dolichol were deposited. When the experiment was tried with solanesol, small crystals were obtained which differed from those of thiourea and also from those of the inclusion compound of squalene. A 'blank experiment' was performed in which the benzene contained no solute. This yielded crystals of thiourea.

If it is accepted that solanesol, like squalene, formed an inclusion compound whereas dolichol clearly did not, these results are consistent with dolichol's containing *cis* isoprene units (see Dicker & Whiting, 1956).

Absence of an ether linkage in dolichol. An ether linkage in such a large molecule might prove difficult to locate by infrared analysis and elementary analysis might just have allowed the presence of one extra oxygen atom in the molecule. Dr D. McHale (Vitamins Ltd.) offered to examine dolichol for the presence of ether linkages by a method with which he was well acquainted:



Perhydrodolichol was refluxed with hydrogen bromide in acetic acid. The product was extracted and refluxed with potassium acetate in acetic acid and the resulting oil, after extraction, was saponified. The product of saponification was refluxed with *p*-phenylazobenzoyl chloride in pyridine and an ester isolated. The migration of this ester on paper impregnated with petroleum jelly, with acetone as solvent, was identical with that of perhydrodolichol *p*-phenylazobenzoate. Had there been an ether linkage in dolichol this treatment

would have been expected to yield two esters, both shorter in chain length than that of perhydrodolichol and therefore more polar. An increase in polarity should have been revealed by chromatography. It was therefore concluded that there was not an ether group in dolichol.

Paper chromatography. The migration of dolichol, solanesol and the C₅₀ isoprenologue of solanesol, namely spadicol (Pennock *et al.* 1963), was studied in different paper chromatographic systems. By extrapolating R_M values (R_M is $\log(1/R_F - 1)$) for solanesol (all-*trans*-nonaprenol) and spadicol (all-*trans*-decaprenol) the approximate value of R_M expected for the hypothetical solanesol isoprenologue with 100 carbon atoms (all-*trans*-eicosaprenol) was obtained. Comparison of this value with that of dolichol showed that the latter compound was in fact much more polar than the hypothetical solanesol analogue. Dr J. Green and Dr D. McHale (Vitamins Ltd.) provided accurate quantitative results for the system which employed acetone as mobile phase and paper impregnated with petroleum jelly as stationary phase (Table 8). This work was done before we were aware of the configuration of the double bonds in dolichol, and the reason for the large difference in R_M values was not then understood. It seems now that although the presence of a saturated isoprene residue would be expected to decrease the R_M of dolichol, relative to a fully isoprenoid alcohol of the solanesol series, the fact that the isoprene units in dolichol are almost all in the *cis* configuration causes an increase in R_M that more than compensates for this.

Possibility of hepene's being the same compound as dolichol. Channon & Marrian (1926) isolated a compound from the livers of pigs, as well as from livers of other animals, which they considered to be an unsaturated hydrocarbon closely related to squalene. The results of further work led to the conclusion that the compound had the formula C₄₅H₇₆ or C₅₀H₈₄ (Channon, Devine & Loach, 1934). The hydrocarbon, which was later named hepene (Dimter, 1941), was present in amounts of 290 mg./kg. of pig liver.

Table 8. *Paper-chromatography data for polyisoprenoid alcohols*

Paper impregnated with petroleum jelly was used as stationary phase and acetone (saturated with petroleum jelly) was used as mobile phase. R_M is $\log(1/R_F - 1)$.

Compound	R_M	R_F
all- <i>trans</i> -Nonaprenol (solanisol)	-0.658	0.82
all- <i>trans</i> -Decaprenol (spadicol)	-0.516	0.77
Solanisol - spadicol	-0.142*	0.05†
all- <i>trans</i> -Eicosaprenol (hypothetical)	+0.904	0.11
Dolichol (from pig liver and human kidney)	+0.342	0.31

* Result is ΔR_M .

† Result is ΔR_F .

We were able to isolate squalene (10–20 mg./kg.) but unable to detect the presence of hepene in the unsaponifiable lipid of pig liver. In fact it appears that dolichol may well have been the compound isolated by Channon's group. The concentration of hepene falls in the range found for dolichol (100–400 mg./kg. of pig liver). Without infrared evidence (or that of nuclear magnetic resonance) it would have been very easy to mistake dolichol for a hydrocarbon, and there was no reason why chemical tests for the presence of a hydroxyl group should have been carried out. Channon *et al.* (1934) purified hepene by chromatography on alumina. It is well known that alumina from different sources may function very differently when used as an adsorbent for chromatography. Thus with columns of the alumina (P. Spence and Sons Ltd., Widnes, Lancs.; acid-washed Brockmann grade 3) used now in this Laboratory dolichol is held more strongly than is ubiquinone-50, whereas it is held less strongly than ubiquinone-50 by the alumina (Brockmann grade 1 with 7% of water added; Giulini, Ludwigshafen, Germany) (U. Gloor, personal communication), i.e. dolichol behaves more like a hydrocarbon than an alcohol on the latter alumina.

Hepene was characterized by its reactions with hydrogen chloride and with bromine. Analysis of the products gave a value for the unsaturation of the hydrocarbon and indicated the lack of an oxygen atom. However, the analysis of dolichol showed that the percentage by weight of oxygen in the molecule is very low and it requires only a trace of impurity to be present or a slight error in analysis to give a figure for oxygen very close to zero. The high iodine value of hepene and its ease of reacting with dry hydrogen chloride are properties shared with dolichol.

Therefore, in view of our failure to isolate hepene from pig liver and our success in isolating an isoprenoid alcohol of similar properties and in similar yield, it seems reasonable to conclude that the compound previously called hepene was in fact the polyisoprenoid alcohol, dolichol.

Biochemistry of dolichol. Little is known about the possible function of dolichol. The alcohol has been found to be distributed fairly evenly in cell fractions of pig liver (Burgos & Morton, 1962). This may indicate a structural role but we could not reconcile this with the virtual absence of the alcohol from some tissues. Most of the compound in pig liver is esterified (P. H. W. Butterworth, unpublished work) and it could be acting as a reserve of isoprene units or possibly as the result of attempts at detoxication of isoprenoid compounds. Preliminary results (H. H. Draper & P. H. W. Butterworth, unpublished work) indicate that [¹⁴C]mevalonate is incorporated into pig-liver dolichol *in vivo*.

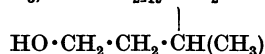
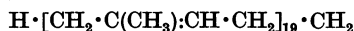
This is the first report of the origin in animal tissues of a compound containing predominantly *cis* isoprene units. To our knowledge the only other natural material of this type is *Hevea* rubber.

SUMMARY

1. The isolation and purification of dolichol from pig liver and from human kidney are described.

2. The equivalent weight of the alcohol was found to be near 1380. Mass spectrometry and depression of the freezing point of cyclohexane showed that this was also the molecular weight.

3. Infrared analysis indicated that the alcohol was isoprenoid and contained mainly *cis* isoprene units. Nuclear magnetic resonance confirmed this and the spectrum was that expected of a compound with the structure



containing 15 or 16 internal isoprene units in the *cis* configuration.

4. Iodine value, hydrogen uptake and ultra-violet absorption were essentially in agreement with the presence of 19 double bonds/molecule.

5. Ozonolysis of the *p*-nitrobenzoate yielded acetone, laevulinaldehyde and 4-methyl-6-*p*-nitrobenzoylhexanoic acid, as expected.

6. The alcohol showed slight optical activity.

7. Attempts to form a thiourea inclusion compound and to detect the presence of an ether linkage failed.

8. A possible explanation for the apparently anomalous mobility of dolichol on paper chromatograms is considered.

9. The possibility is discussed that hepene, previously regarded as a hydrocarbon, was really dolichol.

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The Role of Glucose and Acetate in the Oxidative Metabolism of the Testis and Epididymis of the Ram

By E. F. ANNISON*

Department of Biochemistry and Nutrition, University of New England, Armidale

AND T. W. SCOTT

Department of Veterinary Physiology, Sydney University

AND G. M. H. WAITES

Commonwealth Scientific and Industrial Research Organization, Division of Animal Physiology, The Ian Clunies Ross Animal Research Laboratory, Prospect, New South Wales, Australia

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Information on the metabolism of ejaculated spermatozoa is extensive (Mann, 1954), but little is known about the metabolic activity of the testis in which the spermatozoa are formed or of the epididymis in which they mature and are stored. Studies with tissue slices have failed to reveal differences in the metabolic rate of these structures (T. Mann, cited by Cross & Silver, 1962). An attempt has been made to investigate the metabolism of the testis and epididymis *in vivo* by using catheterization techniques that have been successfully applied to the study of other organs, e.g. heart (Bing *et al.* 1953), liver (Kolodny, Kline & Altszuler, 1962), brain (Sacks, 1956) and kidney (Levy, 1962). Most studies have been based on estimates of the net uptake of a particular substrate by the organ. These are calculated from the arteriovenous differences of

* Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford.

that substrate, and the total oxidative activity of the tissue under investigation may be calculated from blood-gas exchanges measured at the same time. In the present studies glucose and acetate metabolism by the testis and epididymis of the anaesthetized ram were examined under controlled temperature conditions by measurements of arteriovenous differences and also by the use of ¹⁴C-labelled substrates. These were continuously infused to achieve constant specific radioactivities of substrate in the circulation, when arteriovenous differences in the concentration and specific radioactivity of the CO₂ of the blood passing through the testis and epididymis allowed the direct contribution of substrate to the oxidative metabolism of the tissues to be calculated.

A preliminary report of this work was presented to the Biochemical Society (Annison, Scott & Waites, 1962).