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The Intracellular Distribution of Dolichol in Pig Liver

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Rowland, Latimer & Giles (1956) reported the isolation from tobacco of solanesol, a long-chain isoprenoid alcohol. Solanesol was originally thought to be a primary alcohol, $C_{60}H_{81}OH$, consisting of ten isoprenoid residues, but it has since been shown to have the formula $C_{45}H_{73}OH$ (Erickson, Shunk, Trenner, Arison & Folkers, 1959; Rüegg *et al.* 1960). When searching for solanesol in animal tissue, Hemming, Morton & Pennock (1960) found a compound which showed no selective absorption in the ultraviolet region but had an infrared spectrum closely resembling that of solanesol.

Pennock, Hemming & Morton (1960) showed that the new compound was a long-chain isoprenoid alcohol with 20 isoprene residues and a primary alcohol group. The complete chemical structure has not yet been established, but the compound was shown to be $C_{100}H_{161}OH$, and the name dolichol was proposed. Dolichol has been found in several animal tissues in different quantities and small amounts have been found in plants. Dolichol-like material has been found in baker's yeast (J. Burgos, unpublished work) and dolichol was also isolated from *Aspergillus fumigatus* (N. M. Packter & J. Glover, unpublished work). The physiological function of dolichol is still unknown, and it was thought that its distribution among different animal and plant tissues and in the various cell fractions would be a great help in any attempt to establish its role.

The present paper deals with the intracellular distribution of dolichol in pig liver, which contains the alcohol in relatively high concentration.

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EXPERIMENTAL

Cell fractionation. Pig liver was obtained from the Stanley Abattoir, Liverpool, as soon as possible after slaughter of the animal, and was transported in an ice-cold Thermos flask. After removal of fat and connective tissue the liver was minced with scissors and forced through a stainless-steel sieve (20 mesh) and the pulp was collected. A portion of the pulp was taken as a control and the remainder was homogenized and submitted to a scheme of differential centrifuging based on that of Schneider & Hogeboom (1950). During these procedures the temperature was kept between 0° and 5°.

The sieved pulp was disrupted in a Potter-Elvehjem homogenizer in 5 g. batches, 10 ml. of 0.25 M-sucrose being used each time. Each batch was homogenized for about 90 sec. at full speed, the pestle being moved up and down five times during this period. The cell debris and nuclei were sedimented in a refrigerated centrifuge (Measuring and Scientific Equipment Ltd.) at 900g for 15 min. The precipitate was resuspended in sucrose and rehomogenized in the Potter-Elvehjem apparatus and then centrifuged as before. This process was then repeated with a less rigorous homogenization. The supernatant fractions were bulked together.

The mitochondria were sedimented at 7500g for 20 min. and the precipitate was resuspended in sucrose solution and centrifuged once more. The microsome precipitate was obtained by centrifuging at 20 000g for 20 min. The temperature was kept between 0° and 3° during these operations. The supernatant fraction was heated in a boiling-water bath for about 15 min., the protein being denatured and separated by centrifuging at speed 10 (3500g) in an International centrifuge for 30 min. to give a 'groundplasm' precipitate and another supernatant fraction.

Protein estimation. A small portion of each cell fraction was used for protein determination. The method adopted was the biuret quantitative reaction (Cleland & Slater, 1953) and the extinction at 540 mμ was measured in a

Unicam SP. 600 photoelectric spectrophotometer, after filtration of the reaction mixture. A standard curve was prepared, with fat-free casein as reactant.

Preparation of unsaponifiable material. The four cellular fractions and the tissue control were digested by adding 1 ml. of methanolic 0.25% pyrogallol and 0.5 ml. of aq. 60% (w/v) KOH for each g. (wet wt.) of tissue. The mixture was refluxed for 45 min. After cooling, water (3 vol.) was added, and the solution was extracted four times with ether. The combined extracts were washed with water until free from alkali, dried over anhydrous Na_2SO_4 and filtered through a sintered-glass funnel. The filtrate was evaporated to dryness, finally under nitrogen.

Chromatography on alumina. The unsaponifiable lipids were chromatographed on Brockmann grade-3 acid-washed alumina, obtained as follows. Grade O alumina (P. Spence and Sons Ltd., Widnes, Lancs.) was treated with N-HCl overnight and was then washed with water until free from chloride. The alumina was dried overnight at 115° and water was added as required, in amount (ascertained by trial) so that ubiquinone could be eluted from a column with 6% of ether in light petroleum (b.p. $40-60^\circ$). The weight of alumina used was 1 g./mg. of unsaponifiable matter in each fraction, the size of the column being adjusted to the weight of adsorbent, e.g. 3.5×10 cm. for 100 g. The following fractions were collected: 2, 4, 6, 7, 8, 10, 12 and 20% of ether in light petroleum followed by a final elution with ether. The volume of eluent was normally 1 l./100 g. of adsorbent. The ultraviolet-absorption spectrum of each fraction was examined and where possible its infrared absorption was determined. Ubiquinone was usually found in the 6% ether-light petroleum fractions with small amounts in the 4 and 7% ether-light petroleum fractions, whereas dolichol was present in the 6 and 7% ether-light petroleum fractions. The dolichol-containing fractions were bulked and acetylated, dolichyl acetate and ubiquinone being more readily separable than free dolichol and ubiquinone.

Acetylation. Samples weighing between 10 and 20 mg. were dissolved in pyridine (0.5 ml.), and acetic anhydride (3 ml.) was added. The mixture was left overnight at room temperature and was then poured into ice-cold 5N-HCl (10 ml.) and extracted three times with 50% ether-light petroleum. The combined extracts were washed with 5N-HCl and then with water until free from chloride. The solvent was removed and the extract chromatographed on Brockmann grade-3 acid-washed alumina. Usually the fractions eluted with light petroleum, 1, 2 and 3% ether-light petroleum and also ether were collected; the dolichyl acetate was found in the 1 and 2% ether-light petroleum fractions.

Saponification of the acetate. Dolichyl acetate-containing fractions were saponified by dissolving them in a minimal amount of ether (usually 0.25 ml.), adding 4 ml. of ethanol and 0.5 ml. of aq. 60% KOH and boiling for 10 min. Water was added, and the solution cooled and extracted three times with 50% ether-light petroleum. After removal of the solvent the extract was chromatographed on alumina and the dolichol fraction collected.

Thin-plate chromatography. The final purification of dolichol was achieved by thin-layer adsorption chromatography on silica gel according to Stahl (1959). The dolichol-containing fraction was applied as a line, in cyclohexane, and authentic dolichol was spotted on the plate at each end of the line. The developing solvent used was 1% (v/v) of

methanol in benzene and the chromatography was complete in 90 min. The authentic dolichol spots were developed by spraying each end of the plate with a solution of 0.5% of iodine in chloroform. Iodine was 'dissolved' by the lipid to give a brown spot. The silica gel located between the two dolichol markers was scraped off the plate and extracted three times with ether, the ethereal solution being removed by means of a filter-stick. The solvent was evaporated under a stream of nitrogen and then vacuum-desiccated. The infrared spectrum of the extracted lipid agreed with that of dolichol. The weight of dolichol obtained from each cellular fraction was recorded as that recovered after thin-plate chromatography.

Spectrophotometry. The ultraviolet-absorption spectra were measured in a Unicam SP. 500 photoelectric spectrophotometer with cyclohexane as solvent. Ubiquinone was estimated by borohydride reduction in ethanol (Crane, Lester, Widmer & Hatefi, 1959). The presence of dolichol in a fraction was detected by certain absorption bands in the infrared spectrum. The main bands in the infrared region used for identifying dolichol were 2.94μ (OH); 6.0μ (unconjugated double bonds); 9.4μ (C-OH); 11.2 , 11.9 , 13.4μ (all-trans-isoprene). Dolichyl acetate showed no band at 2.94μ but had new bands at 5.72 and 8.08μ for carbonyl and C-O absorption respectively.

Solvents. Ether was dried over sodium wire and before use was freshly redistilled over reduced iron. Light petroleum ($40-60^\circ$) was dried over sodium wire and redistilled. Spectroscopically pure ethanol was prepared by refluxing for 6 hr. over zinc dust and NaOH and then distilling.

RESULTS AND DISCUSSION

Pig liver (200 g.) yielded 176 g. of sieved pulp, 50 g. of which was used as a control. The remaining 126 g. was homogenized and put through the outlined scheme of differential centrifuging. The wet weight of each precipitate and the amount of protein present are given in Table 1. The recovery from the fractionation in terms of protein compared with the control is 99%. Each cellular fraction was saponified and the unsaponifiable lipids were obtained as shown in Table 1.

After chromatography of the unsaponifiable lipids on alumina, ubiquinone and dolichol were estimated and the results are given in Table 2. The recovery of ubiquinone was 97.7% and that of dolichol was apparently 109.2%, compared with control tissue. The total ubiquinone content of pig liver, $34.2\mu\text{g./g.}$, is in good agreement with the figure reported by Cunningham & Morton (1958). The dolichol content of pig liver is $110\mu\text{g./g.}$ The distribution of ubiquinone in the cellular fractions is similar to that reported by Hemming, Pennock & Morton (1958) and Sastry, Jayaraman & Ramasarma (1961) in rat liver. The ubiquinone is predominantly found in the mitochondria and the relatively high concentration in cell debris may be due to unbroken cells. The ubiquinone determinations thus provide a check on the cell fractionation.

Table 1. *Unsaponifiable matter and protein contents of cell fractions obtained by a three-stage Schneider-Hogeboom fractionation of pig-liver homogenate*

Cell fraction	Wet. wt of precipitate (g.)	Protein (%)	Total protein (mg.)	Wt. of unsaponifiable matter (g.)
Cell debris and nuclei	53.5	7.32	3.88	0.0940
Mitochondria	39.5	10.90	4.31	0.1039
Microsomes	52.4	5.74	2.96	0.1602
Groundplasm	131.0	10.37	10.70	0.3155
Whole-tissue control	50.0	17.46	8.73	0.3580

Table 2. *Distribution of ubiquinone and dolichol in cellular fractions derived from pig-liver homogenate*

Cellular fraction	Total ubiquinone (mg.)	Ubiquinone as % of amount in whole tissue	Total dolichol (mg.)	Dolichol as % of amount in whole tissue	Dolichol (mg./g. of protein)
Cell debris and nuclei	1.16	27.0	5.2	37.6	1.34
Mitochondria	2.31	53.6	4.6	32.6	1.07
Microsomes	0.33	7.8	2.3	16.3	0.78
Groundplasm	0.44	9.3	3.2	22.7	0.30
Whole-tissue control	1.71	100	5.5	100	0.65

The dolichol appears to be fairly evenly distributed through the cell fractions and is not concentrated in any one. This fact is more evident when values of dolichol/g. of protein are compared (see Table 2). The groundplasm fraction appears to have less dolichol/g. of protein than the other cellular fractions but even so it contains over one-fifth of the total dolichol.

The accuracy of the estimation of dolichol depends to a large extent on the degree of purification attained for the dolichol-containing fractions. Thin-plate chromatography has proved to be a useful tool for removing many impurities remaining after the preliminary concentration processes.

The present results make it difficult to think of dolichol having other than a structural role. It will be necessary nevertheless to obtain more information about the distribution of dolichol in tissues and cellular fractions before the question of physiological function can be settled.

SUMMARY

1. The dolichol content of several subcellular fractions from pig liver has been studied by preparing the unsaponifiable lipids, chromatography on alumina, acetylation of the dolichol fraction and purification on alumina of the acetate. Saponification of the acetate followed by chromatography on columns of alumina and thin layers of silica gel (Stahl, 1959) gave pure dolichol fractions.

2. Total protein and ubiquinone were also estimated.

3. Pig liver contains 110 mg. of dolichol/kg. of wet tissue.

4. Dolichol is fairly evenly distributed between the different cellular fractions at approximately 1 mg./g. of protein.

5. Ubiquinone distribution in pig liver is very similar to that found in rat liver, since it is mainly concentrated in the mitochondria.

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