Synthesis of Radioactive Dolichol from [4S-³H]Mevalonate in the Regenerating Rat Liver

By H. G. MARTIN* and KAREEN J. I. THORNE†

Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council,
Cambridge, CB4 1XJ, U.K.

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The dolichol of rat liver was labelled by injecting [4S-³H]mevalonate, the precursor of *cis*-isoprene residues, into partially hepatectomized animals. The optimum conditions for labelling the dolichol were to inject the animals with radioactive mevalonate 48 h after hepatectomy and to kill them 12 h later. The concentration of radioactive dolichol was five times as great in regenerating rat liver as in normal liver. The highest concentration of radioactive dolichol was found in the crude mitochondrial and nuclear-debris fractions of the cell. The crude microsomal fractions also contained radioactive dolichol, but at a lower concentration.

Dolichols, the isoprenoid alcohols of eukaryotes, contain 16–22 isoprene units, with a saturated α residue (Pennock *et al.*, 1960; Burgos *et al.*, 1963). They are usually recognized by their characteristic mass spectra (Gough & Hemming, 1970) and are assayed gravimetrically after purification (Burgos & Morton, 1962). We have now developed methods for producing radioactive dolichol *in vivo* in rat liver to make it more easily recognizable in investigations of its function. When [2-¹⁴C]mevalonate is incorporated into lipid by mammalian liver most of the radioactivity appears in sterol and ubiquinone, and little in prenol. This problem was lessened by using mevalonate labelled with ³H in the 4S position. 4S-³H is retained in the biosynthesis of the *cis*-isoprene units of which dolichol is largely composed (Gough & Hemming, 1970), but is lost in the biosynthesis of the *trans*-isoprene units, which predominate in cholesterol and ubiquinone (Cornforth *et al.*, 1966). The mevalonate was injected into partially hepatectomized rats so as to be incorporated into the new lipid synthesized by the regenerating liver.

Experimental

Radioactive mevalonate

[3R,(4S)-4-³H,3S(4R)-4-³H]Mevalonic acid lactone (250 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

* Present address: H. J. Heinz Co. Ltd., Food and Nutrition Research Laboratory, Hayes Park, Hayes, Middx. UB4 8AL, U.K.

† Present address: Strangeways Research Laboratory, Wort's Causeway, Cambridge CB1 4RN, U.K.

Partial hepatectomy

Male and female Hooded rats (weight 250–350 g) were fed from weaning on the stock 41B diet (E. Dixon and Son, Ware, Herts., U.K.). At 5 days before experimentation 1% (w/w) cholesterol was added to the diet to inhibit cholesterol biosynthesis. Surgery, under light diethyl ether anaesthesia, involved the ligation and excision of the large median and left lateral liver lobes, some 60–70% of the total liver weight, leaving the right lateral and caudate lobes to regenerate.

Subcellular fractionation

Regenerating liver samples were rinsed with ice-cold 0.9% NaCl, blotted dry with filter paper, weighed and combined into 15 g batches for homogenization with 2 vol. of 0.25 M-sucrose containing 2 mM-mercaptoethanol. Four batches were combined and made up to 200 ml with 0.25 M-sucrose and 2 mM-mercaptoethanol. All operations were carried out at 0–5°C. The rat liver homogenate was fractionated by a scheme of differential centrifugation based on the method of Schneider & Hogeboom (1950) for the preparation of nuclear debris and mitochondria, and the method of Bergstrand & Dallner (1969) for microsomal fractions and particle-free cytosol. Subcellular fractions were not characterized enzymically and the degree of cross-contamination therefore is not known. In particular the mitochondrial fraction could be contaminated with lysosomes. Samples were taken from each fraction during purification for dolichol and protein determination.

Dolichol purification

Tissue fractions were saponified by heating under reflux for 60–90 min in methanol–60% (w/v) KOH (2:1, v/v). Tissue homogenates and suspensions of subcellular fractions were decreased in volume by freeze-drying before saponification. The unsaponifiable lipid was extracted with diethyl ether, washed with water until free of KOH, and dried by passage through Na_2SO_4 . Preliminary fractionation of the unsaponifiable lipid commenced with the removal of sterol by digitonin precipitation. The lipid was dissolved in 5–10 ml of methanol to which was added an equal volume of a solution of 3% (w/v) digitonin in 95% (v/v) methanol. After 16 h at room temperature the sterol-low lipid was extracted from the precipitated digitonide three times with an equal volume of light petroleum (b.p. 60–80°C). Sterol-low lipid was fractionated on columns of Brockmann grade III alumina (M. Woelm, Eschwege, Germany) by the method of Butterworth *et al.* (1966b), first as the free lipid and again after acetylation. Dolichol could be recovered from the dolichol acetate by saponification.

The authenticity of the dolichol fraction was checked by t.l.c. on silica gel G with ethyl acetate–hexane (1:9, v/v), where free dolichol had R_F 0.36, and with chloroform, where dolichol had R_F 0.70, and by i.r. spectroscopy (Pennock *et al.*, 1960). The molecular weight was determined by acetylation with radioactive acetic anhydride (Thorne & Kodicek, 1966) and was 1409, which compares favourably with the molecular weight of a dolichol containing 20 isoprene units of 1382 (Burgos *et al.*, 1963).

Measurement of radioactivity

Radioactive lipids were dissolved in 10 ml of toluene containing (per litre) 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (Packard Instrument Co., Downer's Grove, Ill., U.S.A.). Radioactivity was measured in low-potassium glass vials in a Packard Tri-Carb 3375 liquid-scintillation spectrometer. All radioactivity measurements were corrected for quenching and expressed as d.p.m.

DNA and protein analyses

Protein was measured by the Folin–Lowry method (Lowry *et al.*, 1951) with bovine plasma albumin as standard (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.). DNA was determined with diphenylamine (Schneider, 1957).

Results and Discussion

Characteristics of liver regeneration

The removal of 65–70% of the liver by excision of the large median and left lateral liver lobes induced very rapid growth in the remaining right lateral and

caudate lobes, which hypertrophied in proportion to their original relative size until the original organ mass was replaced. Growth exceeding that of embryonic or malignant cells completely restored the liver weight within 2 weeks (Fig. 1a). Synthesis of DNA was very

Fig. 1. Time-course of liver regeneration: changes in (a) wet wt., (b) DNA concentration and (c) protein concentration after partial hepatectomy

Percentage increase in wet wt. of regenerating liver was obtained from the formula:

$$R = \frac{[(\text{body wt.} \times 4.85/100) - y] \times 100}{y}$$

Where R is the wet wt. of regenerated liver and y is the wet wt. of liver removed by hepatectomy (Donaldson, 1924). In (b) and (c) dotted lines and arrow indicate range in normal liver.

Table 1. *Incorporation of [4S-³H]mevalonate at different injection times after partial hepatectomy*

Five pairs of rats were injected intraperitoneally with 5 μ Ci of [4S-³H]mevalonate in 0.1 ml of sterile 0.9% NaCl, each, 12, 24, 36, 48 or 72 h after hepatectomy. One of each pair was killed 24 h (Expt. 1) and the other 48 h (Expt. 2) after injection. Preparation of the dolichol fraction is described in the Experimental section.

Injection time (h)	Regenerated liver wet wt. (g)	Total protein (g)	Radioactivity of dolichol fraction (d.p.m.)
Expt. 1			
12	6.01	1.27	472
36	5.34	0.95	912
48	7.99	1.37	3500
72	10.08	1.79	1161
Expt. 2			
12	5.34	1.60	669
24	6.21	1.07	1863
36	7.38	1.55	1600
48	9.82	1.70	2550
72	12.08	2.35	1800

rapid during the first 24 h (Fig. 1b). Maximum protein synthesis occurred on the third day (Fig. 1c). This pattern of compensatory liver growth is as described by Bucher (1963) and Leduc (1964). The present work was performed with regenerating liver samples obtained 2–3 days after hepatectomy. The tissue would therefore be at the stage of active cell growth.

Effect of regeneration time on [4S-³H]mevalonate incorporation

Two groups of experiments were done to determine the optimum conditions for incorporation of radioactive mevalonate into dolichol. First [4S-³H]mevalonate was injected into five pairs of rats at 12, 24, 36, 48 or 72 h after hepatectomy (Table 1). One rat of each pair was killed 24 h after injection and its partner was killed after 48 h. Secondly, when the best time had been selected for mevalonate injection, the animals were killed at different times after injection (Table 2). Maximum incorporation of mevalonate into dolichol was seen when animals were injected 48 h after hepatectomy and killed 12 h later.

Comparison of incorporation of [4S-³H]mevalonate into dolichol by normal and regenerating rat liver

As shown in Table 3 the radioactivity incorporated into dolichol was four to five times as great in regenerating as in normal liver, whether considered per wet wt. of tissue, per μ g of DNA or per mg of protein. This is therefore a useful technique for increasing the incorporation of mevalonate into dolichol.

Table 2. *Incorporation of [4S-³H]mevalonate at different times between injection and killing*

Each of six rats was injected intraperitoneally with 5 μ Ci of [4S-³H]mevalonate in 0.1 ml of sterile 0.9% NaCl 48 h after partial hepatectomy. One rat was killed after each stated time-interval. Preparation of the dolichol fraction is described in the Experimental section.

Time between injection and killing (h)	Regenerated liver wet wt. (g)	Total protein (g)	Radioactivity of dolichol fraction (d.p.m.)
2	7.19	1.40	58.5
4	6.66	1.38	425
8	8.46	1.92	603
12	6.32	1.34	1374
24	6.63	1.24	967
48	8.66	1.82	556

Specificity of incorporation of [4S-³H]mevalonate into dolichol

Only one-tenth of the radioactivity incorporated from [4S-³H]mevalonate into unsaponifiable lipid was found in dolichol. Much of the radioactivity of the other nine-tenths was characterized by column chromatography and t.l.c. as sterol, with some contribution from hydrocarbon and ubiquinone. It is evident that injected [4S-³H]mevalonate is not found only in *cis*-isoprenoid compounds. There appeared to be some isomerization resulting in retention of ³H in *trans*- as well as *cis*- isoprenoid residues. Gough & Hemming (1970) found similar incorporation of ³H from [4S-³H]mevalonate into *trans*-isoprenoid compounds. It is therefore still necessary, even when [4S-³H]mevalonate is used as precursor, to purify the dolichol fraction before determining it. Nevertheless, this represents a considerable improvement over the use of [2-¹⁴C]mevalonate, where 0.2% of the lipid radioactivity is found in dolichol (Butterworth *et al.*, 1966a).

Subcellular distribution

Subcellular fractions of increasing purity are designated 1, 2 and 3 in Table 4. The fractions with the highest contents of radioactive dolichol were the nuclear debris and the mitochondria. As the fractions were purified the concentration of dolichol per mg of protein increased. The microsomal fraction was initially contaminated with a more radioactive fraction, but it seemed to have a real, albeit low, content of dolichol. The radioactivity of the nuclear-debris fraction was not due to the nuclei, since purification of these organelles by the method of Lawson *et al.* (1969) demonstrated that they only had one-quarter of the

Table 3. *Incorporation of [4S-³H]mevalonate into normal and regenerating rat liver*

Three control and three experimental animals were each injected with 5 μ Ci of [4S-³H]mevalonate intraperitoneally and killed 12 h after injection. Preparation of the dolichol fraction is described in the Experimental section. Values are means $\pm$ S.E.M. for three animals.

	Radioactivity of dolichol fraction			
	(total d.p.m.)	(d.p.m./g wet wt. of liver)	(d.p.m./mg of protein)	(d.p.m./g of DNA)
Normal liver	1030	70.7 $\pm$ 12.6	0.29 $\pm$ 0.05	0.096 $\pm$ 0.027
Regenerating liver	2210	349.6 $\pm$ 128.6	1.53 $\pm$ 0.44	0.342 $\pm$ 0.040

Table 4. *Subcellular distribution of radioactive dolichol formed from [4S-³H]mevalonate in regenerating rat liver*

Each of eight rats was injected with 20 μ Ci of [4S-³H]mevalonate 48 h after partial hepatectomy and killed 12 h later. The preparation and analysis of the subcellular fractions is described in the Experimental section. Fraction 1 was the initial pellet; fraction 2 was washed once; fraction 3 was washed twice.

Cell fraction	Protein (g)	Dolichol (d.p.m./mg of protein)
Homogenate	11.1	11.30
Nuclear-debris 1	5.47	8.43
2	4.01	11.35
3	3.33	17.97
Mitochondria 1	1.42	7.21
2	1.38	14.59
3	0.49	19.30
Microsomal 1	0.68	6.31
fraction 2	0.32	3.38
3	0.21	3.26
Cytosol	3.44	1.07

radioactivity per mg of protein of the original homogenate. It is probably therefore due to mitochondrial contamination. This was observed by examination of the nuclear-debris fraction by phase-contrast light-microscopy. It is possible that the amounts of dolichol measured are an underestimate owing to the presence of dolichol phosphate, which is resistant to saponification. However, dolichol phosphate tends to be hydrolysed by endogenous enzymes unless F⁻ is present (Martin & Thorne, 1974). Since F⁻ was not used in this work much of the original dolichol phosphate would have been converted into free dolichol. The distribution of radioactive dolichol in regenerating rat liver did not differ qualitatively from the distribution of total dolichol in pig liver, measured gravimetrically (Burgos & Morton, 1962; Butterworth & Hemming, 1968), but the difference in radioactivity between the different cell fractions was much more marked. The distribution of dolichol synthesized by 12 h in a regenerating liver therefore reflects the subcellular distribution of total dolichol, but the very

large amount of radioactivity in the mitochondria suggests that this is the site of synthesis. The isoprenoid side chain of ubiquinone is also synthesized in the mitochondria (Momose & Rudney, 1972).

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