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THE BIOSYNTHESIS OF DERIVATIVES OF ALLYLIC ALCOHOLS FROM [2-^{14}C] MEVALONATE IN LIVER ENZYME PREPARATIONS AND THEIR RELATION TO SYNTHESIS OF SQUALENE

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STUDIES on the enzymic synthesis of squalene from mevalonic acid (3:5-dihydroxy-3-methylpentanoic acid) in yeast extracts have revealed a number of new intermediates. 5-Phosphomevalonate,[1,2,3] 5-diphosphomevalonate and 3-methylbut-3-en-1-yl pyrophosphate (isopentenyl pyrophosphate)[4,5,6] have been recognized as successive intermediates in the synthesis of squalene. In addition, Lynen et al.[5] found that a protein fraction of yeast extracts catalysed the synthesis from mevalonate of farnesyl pyrophosphate which was

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[1] T. T. Tchen, J. Amer. Chem. Soc. 79, (1957).

[2] T. T. Tchen, J. Biol. Chem. 233, 1100 (1958).

[3] F. Lynen, Ciba Symposium on Biosynthesis of Terpenes and Sterols p. 95. J. & A. Churchill, London (1959).

[4] S. Chaykin, J. Law, A. H. Phillips, T. T. Tchen and K. Bloch, Proc. Nat. Acad. Sci. Wash. 44, 998 (1958).

[5] F. Lynen, H. Eggerer, U. Henning and I. Kessel, Angew. Chem. 70, 739 (1958).

[6] U. Henning, E. M. Möslein and F. Lynen, Arch. Biochem. Biophys. 83, 259 (1959).

converted to squalene on addition to yeast cell particles and reduced pyridine nucleotides.

Experiments on the same problem with liver enzymes indicate that the reactions of squalene biosynthesis in mammalian cells are similar to those occurring in yeast cells. 5-Phosphomevalonate and 5-diphosphomevalonate and the enzymes responsible for their formation in liver extracts have already been described.[7-11] I wish to report briefly the synthesis by liver enzymes of derivatives of allylic alcohols ("polyprenols"), which have properties similar to those described by Lynen et al.[5] for farnesyl pyrophosphate.

Two types of enzyme preparations were used. One was a protein fraction (F_{30}^{60}-enzymes), precipitable between 30 and 60 per cent ammonium sulphate saturation, of the supernatant of rat liver homogenates[12] centrifuged at 105,000 g for 60 min. This precipitate was dissolved in 0.02 M $KHCO_3$ and dialysed against the same for 3 - 4 hr. to give a protein content of 30 - 50 mg/ml. The second preparation was a suspension of liver-cell microsomes isolated from the homogenates.[13]

When the F_{30}^{60}-enzymes were incubated with DL-[2-^{14}C]mevalonate,

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7 A. de Waard, Doctoral Thesis: Investigations on the Intermediates in the Biosynthesis of Fatty Acids and Cholesterol, University of Utrecht. Drukkerij Gebr. Janssen, Nijmegen (1958).

8 G. Popják, Ciba Symposium on Biosynthesis of Terpenes and Sterols p. 148. J. & A. Churchill, London (1959).

9 A. de Waard and G. Popják, Biochem. J. 73, 410 (1959).

10 H. R. Levy and G. Popják, Biochem. J. 72, 35P, (1959).

11 H. R. Levy and G. Popják, Biochem. J. Submitted for publication.

12 N. L. R. Bucher and K. McGarrahan, J. Biol. Chem. 222, 1 (1956).

13 G. Popják, L. Gosselin, I. Y. Gore and R. G. Gould, Biochem. J. 69, 238 (1958).

adenosinetriphosphate (ATP) and Mg^{2+} ions aerobically or anaerobically, in the presence of absence of reduced pyridine nucleotides, only traces of radioactivity could be extracted with light petroleum (b.p. 40 - 60°) from the alkaline incubation mixture after saponification with 2 N KOH. On the other hand when the alkaline digests were made acid (pH about 2) with H_2SO_4, ^{14}C-labelled substances could be readily extracted with light petroleum. Although such substances might be thought to be acidic in character, partitioning between aqueous alkali (0.2 N KOH or $KHCO_3$) and light petroleum showed that only 10 - 15 per cent of these was acidic and the larger fraction 85 - 90 per cent, neutral in character. The results of a typical experiment are shown in Table 1, which also demonstrate that both ATP and Mg^{2+} ions are needed for the formation of these substances.

Both the neutral and acidic products (the latter after methylation with diazomethane) were analysed by gas-liquid radiochromatography (GLRC).[14] The neutral products contained several components, which had retention volumes identical with a number of branched chain primary and tertiary alcohols on columns with both polar (ethyleneglycol - adipate polyester) and non-polar (Apiezon-L) stationary phases at 197°C. The following substances were found: dimethylallyl alcohol, linalool, nerol (cis-geraniol), geraniol, nerolidol, trans - trans-farnesol and two further substances with retention volumes expected of the C_{20} analogues of nerolidol (geranyllinalool) and of farnesol (geranylgeraniol). In the specimen of neutral products, pooled from incubation nos. 1, 4, 5 and 6 of the experiment shown in Table 1, 62 per cent of the ^{14}C was present in nerolidol, 19.50 per cent in farnesol

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[14] G. Popják, A. E. Lowe, D. Moore, L. Brown and F. A. Smith, J. Lipid Res. 1, In Press (1959).

TABLE 1. SYNTHESIS OF NEUTRAL AND ACIDIC SUBSTANCES FROM DL-[2-^{14}C] MEVALONATE WITH SOLUBLE ENZYME FRACTION (F_{30}^{60}) OF RAT LIVER

The complete system contained in a final volume of 4 ml: 97.5 mg F_{30}^{60}-protein; 30 μmoles of ATP; 20 μmoles of $MgCl_2$; 400 μmoles of potassium hydrogen phosphate buffer, pH 7.5; 40 μmoles of NaF; 5 μmoles of DL-[2-^{14}C]mevalonate (140,000 counts/min at negligible thickness). Omissions from and additions to this system were made as shown.

Incubation no.	Enzyme system, omissions and additions	^{14}C counts found	
		In neutral products	In acidic products
1.	Complete	30,220	4122
2.	No Mg^{2+}	19	0
3.	No ATP; +ADP (30 μmoles)	2400	214
4.	+TPNH (2 μmoles)	27,690	4018
5.	+TPN (2 μmoles)	25,130	3966
6.	+TPNH + DPNH (2 μmoles each)	25,170	2370

and the remainder nearly equally distributed among the other components mentioned.

It follows from the mode of isolation of these alcohols that they did not occur in the free form, but were derivatives of some kind resistant to alkali but unstable in the presence of acid. Lynen *et al.*[5] noted that farnesyl pyrophosphate, isolated from incubations of yeast extracts with mevalonate, split into inorganic pyrophosphate and an organic residue on addition of 5 per cent trichloroacetic acid. It seems highly probable that the alcohols in the liver enzyme incubations were also present as the

pyrophosphates.

The unhydrolysed derivatives of the polyprenols could be extracted quantitatively from the slightly alkaline (pH 7.5) enzyme incubation mixtures with collidine as was also found for farnesyl pyrophosphate.[5] An aqueous solution of these derivatives could be prepared by shaking the collidine extract with aqueous 0.01 M $KHCO_3$ and ether, the polyprenol derivatives being transferred into the aqueous phase. When a sample of such an aqueous solution (aq.-PP) was acidified to pH 2 the free alcohols could be readily extracted with light petroleum. GLRC revealed the same substances and in the same ratios as were noted when the alcohols were extracted directly from the acidified enzyme incubations.

In view of the instability of esters of allylic alcohols in the presence of acid, one must beware of a rearrangement to a tertiary alcohol during elimination, induced by acid, from the ester of a primary allylic alcohol. Thus farnesyl pyrophosphate may yield not only farnesol but also nerolidol, and geranyl pyrophosphate may give rise to geraniol and to linalool. Elimination from an ester of an allylic alcohol in the presence of acid may occur readily, for the resulting electron deficient species may be partially stabilized by resonance between two mesomeric forms (a) $>C = \underset{|}{C} - \overset{+}{C}<$ and (b) $>\overset{+}{C} - \underset{|}{C} = C<$, which in the presence of water may yield either the primary or the tertiary alcohol.

It was particularly important to know whether the nerolidol found was genuinely an enzymic product, or merely resulted from the rearrangement of an ester of farnesol. A sample of aq.-PP (pH 8.5) after extraction with light petroleum was hydrolysed with snake venom (Naja flava) phosphatases at pH 8.3 in 0.15 M trishydroxymethyl-aminomethane buffer at 37° for 2,

24 and 48 hr. The alkaline incubation mixture was then extracted with light petroleum; analysis of the extracts by GLRC showed only the presence of dimethyl allyl alcohol, geraniol, nerolidol and of farnesol. However, whereas after acidification the amount of nerolidol was usually three times greater than that of farnesol, after the snake venom hydrolysis the amount of farnesol was four to six times greater than that of nerolidol. One further test, suggested by Dr. J. W. Cornforth, was carried out to ascertain whether or not the nerolidol was a genuine enzymic product. A freeze-dried sample of aq.-PP was reduced with $LiAlH_4$ in dry ether as used by Karrer and Jucker[15] for the fission of cetylphosphate. Approximately one-half of the polyprenols, originally bound in the specimen, appeared in the neutral fraction after reduction; these - according to GLRC analysis - consisted of two hitherto unidentified components (?hydrocarbons) and of nerolidol and farnesol; the ratio of the amounts of the latter two being 1:5.

These two tests leave little doubt of the genuine enzymic formation of a nerolidyl ester, most probably the pyrophosphate. The preponderence of nerolidol, and the finding or nerol and linalool in the specimens after release of the polyprenols from their conjugates by acid is no doubt due to the rearrangement discussed earlier. The most probable mechanism of the formation of nerolidyl pyrophosphate is the isomerization of farnesyl pyrophosphate by an intramolecular rearrangement:

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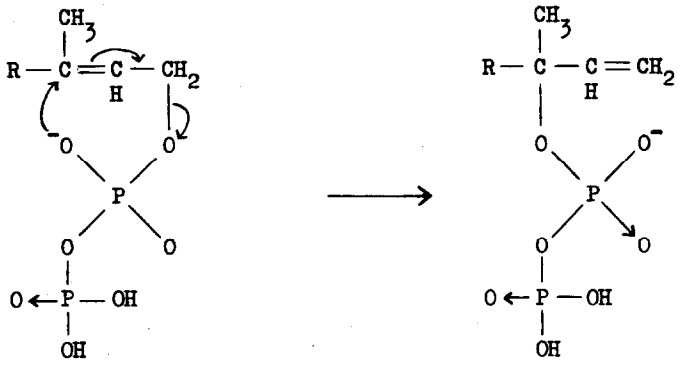
[15] P. Karrer and E. Jucker, Helv. Chim. Acta 35, 1586 (1952).



Further experiments indicated that the polyprenol derivatives are intermediates in the biosynthesis of squalene (and hence of cholesterol), the immediate precursors being, most likely, the farnesyl and nerolidyl esters.

In an experiment, the results of which are shown in Table 2, F_{30}^{60}-enzymes were first incubated on a large scale for 2 hr with DL-[2-^{14}C]mevalonate, ATP and Mg^{2+} ions. During this time about 25 per cent of the DL-mevalonate added was converted into the polyprenol derivatives, a small amount of acidic products being also formed. Several 4 ml samples of this preliminary bulk incubation were then treated in different ways. As is shown in Table 2, the addition of microsomes (0.2 ml suspension), ascorbic acid (30 μmoles) and of TPNH + DPNH (2 μmoles each), followed by incubation for a further 1 hr with air as gas phase, resulted in the formation of squalene, of cholesterol, and of carboxylic acids at the expense of the polyprenols. Analysis by GLRC showed that the farnesol and nerolidol components of the polyprenol mixture were principally involved in the change. In incubation no. 1, Table 2, 25,560 counts out of the 40,980

found in polyprenols were in farnesol + nerolidol; after addition of microsomes, ascorbate and of TPNH + DPNH this was reduced to 8355, a loss of 17,205 counts, of which 16,200 could be accounted for by the squalene and cholesterol synthesized.

In the absence of either ascorbate, or of TPNH + DPNH very little squalene and sterol was formed but the polyprenols were converted into carboxylic acids. The results of Table 2 show further (incubations nos. 3, 4 and 5) that when squalene was not synthesized on account of the omission of one or more essential coenzyme, there was a net loss of ^{14}C from the incubations. This suggests a catabolic disposal of squalene precursors by a mechanism as yet undetermined.

That the carboxylic acids are not intermediates in squalene biosynthesis was indicated by the fact that when these were allowed to accumulate in an incubation the subsequent addition of ascorbate and pyridine nucleotides failed to cause the synthesis of squalene and cholesterol (cf. incubation nos. 5 and 6, Table 2). These carboxylic acids appear to inhibit the utilization of the polyprenol derivatives for the synthesis of squalene.

Analysis of the methyl esters of the acids by GLRC revealed the same components as were noted previously[16] in incubations of whole homogenates and in the unfractionated supernatants of these obtained by centrifugation at 105,000 *g*. These were: trans-geranate, trans - trans-farnesoate (trans - trans-3:7:11-trimethyldodeca-2:6:10-trienoate) and the C_{20} all-trans 3:7:11:15-tetramethylhexadeca-2:6:10:14-tetraenoate. In

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[16] G. Popjak, M. Horning, N. L. R. Bucher and R. H. Cornforth, Biochem. J. 72, 34P (1959).

TABLE 2. UTILIZATION OF POLYPRENOL DERIVATIVES FOR SQUALENE AND STEROL SYNTHESIS AND THEIR CONVERSION INTO TERPENOID ACIDS BY MICROSOMES

First F_{30}^{60}-enzymes were incubated for 2 hr at 37° with ATP and Mg^{2+} ions in a total volume of 60 ml. Each 4 ml of this bulk incubation contained 80 mg of proteins, and the same reagents as shown in Table 1 + 120 μmoles of nicotinamide. At the end of 2 hr 4 ml samples were withdrawn for analysis of products (incubation no. 1) and for setting up of further incubations for another 1 hr with microsomes as described in the text. All data below relate to a volume of 4 ml of the original bulk incubation.

Incubation no.	Additions after preliminary incubation	^{14}C counts found in products			
		Polyprenols	Squalene +sterol	Acids	Total
1.	None; F_{30}^{60}-enzymes alone	40,980	0	990	41,970
2.	Microsomes + ascorbate + DPNH + TPNH	15,150	16,200	13,030	44,380
3.	Microsomes + ascorbate	13,370	750	18,770	32,890
4.	Microsomes + DPNH + TPNH	12,410	2950	8700	24,060
5.	Microsomes	17,280	460	10,160	27,900
6.	Microsomes, and after 3rd hr +ascorbate + DPNH + TPNH	9400	1026	14,170	23,576

addition to these three acids, several others - in smaller amounts - were also found but not identified. Acids are formed only in small amounts by the F_{30}^{60}-enzymes alone, but their yield increases substantially in the presence of microsomes. In the previous communication[16] about the synthesis of terpenoid acids in liver enzyme preparations it was said that

the yields of the acids in the F_{30}^{60}-preparations was as good as in the whole homogenates or in their unfractionated supernatants. This was a mis-statement made in good faith, for it was not appreciated at that time that most of the petroleum-soluble substances released after acidification of the saponified F_{30}^{60}-incubations were neutral in character. In whole homogenates and in unfractionated supernatants of these only small amounts of the polyprenols can be found after 2 - 3 hr of incubation with mevalonate, the acidic products predominating (disregarding squalene and cholesterol synthesized in the whole homogenates).

Identification of the polyprenols, like that of the acids, synthesized from [2-^{14}C] mevalonate in liver enzyme incubations rests at present entirely on the GLRC analysis in which the ^{14}C-labelled substances had retention volumes indistinguishable from those of authentic marker substances, which were cochromatographed with the biosynthetic specimens. The power of resolution of these columns, however, was such that trans - trans (or trans) isomers were well separated from cis - trans (or cis) isomers.

The theoretical implications of the results described are discussed in the accompanying communication.[17]

I am indebted to Dr. M. Stoll of Firmenich et Cie, Geneva, for specimens of farnesol and nerolidol, to Dr. B. C. L. Weeden of the Imperial College of Science and Technology, London, for samples of geraniol, nerol, methyl-trans-geranate and methyl-cis-geranate, to Dr. Rita H. Cornforth of the National Institute for Medical Research, London, for farnesoic acid, and to Dr. Karl Folkers of Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey, U. S. A., for a specimen containing the isomers of 3:7:11:15-tetramethylhexadeca-tetraenoic acid.

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[17] J. W. Cornforth and G. Popják, Tetrahedron Letters No. 19 29 (1959).