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Studies on the biosynthesis of cholesterol: XIV. the origin of prenoic acids from allyl pyrophosphates in liver enzyme systems

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SUMMARY

Allyl pyrophosphates (3,3-dimethylallyl, geranyl, and farnesyl pyrophosphate), which are known intermediates in the biosynthesis of squalene from mevalonate, are also metabolized in the liver by an alternative pathway to acids. The first step in the conversion of the allyl pyrophosphates into the acids (dimethylacrylic, geranoic, and farnesoic acids) is their irreversible dephosphorylation into free prenols by a microsomal phosphatase, which has its optimum pH at around 7.0. In a second step the free prenols are irreversibly dehydrogenated to the acids by liver alcohol dehydrogenase and aldehyde dehydrogenase present in a soluble protein fraction of liver homogenates. This dehydrogenation proceeds best at pH 7.5 and is inhibited by sulfhydryl reagents. The prenols are more specific substrates for liver alcohol dehydrogenase than is ethanol, but they cannot act as substrates for yeast alcohol dehydrogenase. The formation of allyl pyrophosphates, of free prenols, and of prenoic acids from mevalonate was also observed *in vivo*.

The biosynthesis of farnesoic acid[1] from mevalonate-2-C^{14} in liver enzyme preparations, synthesizing squalene and cholesterol, was first reported by Dituri *et al.* (1), and was confirmed in this laboratory (2, 3). Gas-liquid radiochromatographic analysis (4) of acids isolated from enzyme incubations revealed, however, that *trans-trans*-farnesoic acid was only one of several acids formed from mevalonate-2-C^{14} (3). Our interest in the biological synthesis of farnesoic acid from mevalonate was concerned with two possibilities, viz. (*a*) that this substance might be a precursor of squalene, as was suggested by Dituri *et al.* (1), or (*b*) that it originated by an alternative pathway of the metabolism of intermediates of squalene biosynthesis. The incorporation of C^{14} from biosynthetically prepared C^{14}-farnesoic acid into cholesterol, reported by Dituri *et al.* (1), could not be confirmed in our laboratory (3), and farnesoic acid was therefore excluded as an intermediate in squalene biosynthesis. Since the pyrophosphates of dimethylallyl alcohol, of geraniol, and of farnesol ("allyl pyrophosphates") had been identified as intermediates in the synthesis of squalene, both in yeast and in liver enzyme systems (5 to 9), it seemed most probable that the acids synthesized from mevalonate in liver enzyme preparations were in fact products of an alternative metabolism of these intermediates, as has already been suggested (3).

It will be shown in this paper that dimethylacrylic, geranoic, and farnesoic acids are formed in liver enzyme preparations from allyl pyrophosphates by a combined action of a phosphatase, present mainly in the microsomes, and of two dehydrogenases.

METHODS AND MATERIALS

Enzyme Preparations. Soluble supernatants and microsomes were prepared as described previously (10, 11) from rat liver homogenates made according to Bucher and McGarrahan (12). A fraction of the soluble supernatant, F_{30}^{60}, was a preparation similar to that described previously, and shown to synthesize the pyrophosphates of dimethylallyl alcohol, of geraniol, and of farnesol from DL-mevalonate-2-C^{14}, or from (−)-5-phosphomevalonate-2-C^{14}, and ATP in the presence of Mg^{++} (9). In a few experiments, the proteins of the soluble supernatant of rat liver homogenates were precipitated with $(NH_4)_2SO_4$ (90% saturation), and

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[1] This substance has previously been referred to as farnesenic acid. The present term is preferred since the acid should be considered as derived from farnesol.

this unfractionated protein preparation (F_0^{90}) was used —after being dissolved in 0.02 M $KHCO_3$ and dialyzed (11)—as a source of enzymes. In addition to the F_{30}^{60}-enzymes, fractions of the soluble supernatant of homogenates precipitable between 0% and 30%, and between 60% and 90% saturation with $(NH_4)_2SO_4$ (F_0^{30}- and F_{60}^{90}-preparations, respectively) were also tested.

Suspensions of microsomes were stored at −15°, and retained their activity for periods of at least 3 months. In one experiment, microsomes were extracted with Lubrol-W, a nonionic detergent (Imperial Chemical Industries Ltd., England), and a Lubrol-insoluble and a Lubrol-soluble fraction was obtained. The usual suspension of microsomes (11) was diluted with an equal volume of 0.1 M potassium hydrogen phosphate buffer, pH 7.4, and a 10% aqueous solution of Lubrol-W was added to it in small portions up to a final concentration of 1%. The suspension, kept at 0°, was homogenized repeatedly during 30 minutes in a loosely fitting homogenizer; it was then centrifuged at 105,000 × *g* for 1 hour. The sedimented pellet was resuspended in the 0.1 M phosphate buffer containing 1 mM EDTA[2]; the resuspended particles and the Lubrol-soluble supernatant were dialyzed for 3 hours against the same medium. Approximately one-half of the microsomal proteins was rendered soluble by the Lubrol treatment.

DL-*Mevalonic Acid Lactone-2-C*^{14}. This was purchased from the Radiochemical Centre, Amersham, Bucks, England, and was converted to the potassium salt by hydrolysis with a small excess of KOH at 37°. The optically active (−)5-phosphomevalonate-2-C^{14} was prepared with purified liver mevalonic kinase according to Levy and Popják (13).

C^{14}*-Allyl Pyrophosphates.* These were synthesized enzymically from (−)5-phosphomevalonate-2-C^{14} (26,000 cpm/μmole) and purified according to Goodman and Popják (9). After complete hydrolysis of a sample of the allyl pyrophosphates by calf intestinal phosphatase at pH 8.6, the alcohols liberated were extracted with petroleum ether. Gas-liquid radiochromatographic analysis of these alcohols showed that they consisted of 3% dimethylallyl alcohol, 8% geraniol, 11% nerolidol, 74% *trans-trans*-farnesol, and of some substances with retention volumes greater than that of 4% farnesol. Thus the preparation consisted mostly of C^{14}-farnesyl pyrophosphate.

C^{14}*-Prenols.*[3] These were prepared in three different ways from C^{14}-allyl pyrophosphates. In the first procedure, the purified preparation of the allyl pyrophosphates was hydrolyzed with prostatic acid phosphatase (9) and the liberated alcohols (mostly farnesol, see above) extracted with petroleum ether.

In the second procedure, C^{14}-allyl pyrophosphates were first synthesized from DL-mevalonate-2-C^{14} with the F_{30}^{60}-enzymes (9) and then, without isolation, were hydrolyzed by the addition of a suspension of microsomes (0.05 ml/ml of incubation) and of 1 mM PCMB; after 90 minutes of incubation with the microsomes at 37°, the C^{14}-prenols were extracted with petroleum ether. The use of PCMB prevented the conversion of the prenols to acids by the dehydrogenases present in the F_{30}^{60}-preparations (see also under Results and Discussion). Analysis of the C^{14}-prenols by gas-liquid radiochromatography showed them to consist of 2% dimethylallyl alcohol, 12% geraniol, and of 73% *trans-trans*-farnesol, together with a little nerolidol and some other unidentified substances.

In a third procedure, C^{14}-allyl pyrophosphates were hydrolyzed for 16 hours by acid (0.2 N HCl) at room temperature, and the prenols extracted with petroleum ether. It was shown previously (9) that hydrolysis of the allyl pyrophosphates by acid causes an allylic rearrangement, most of the farnesyl pyrophosphate being converted into nerolidol. In the particular preparation, 65% of the radioactivity was associated with nerolidol, 20% with farnesol, and the remainder with C_5, C_{10}, and with some other alcohols.

The C^{14}-prenols were kept (without addition of carrier) in petroleum ether at −15°; when needed as substrates in enzyme incubations, samples of their solution were slowly evaporated under a stream of N_2 in the incubation vessel.

Incubations. Most of the incubations (1 ml) were done at 37° in 10-ml glass-stoppered centrifuge tubes. For "anaerobic" incubations, the tubes were flushed with N_2 and immediately stoppered.

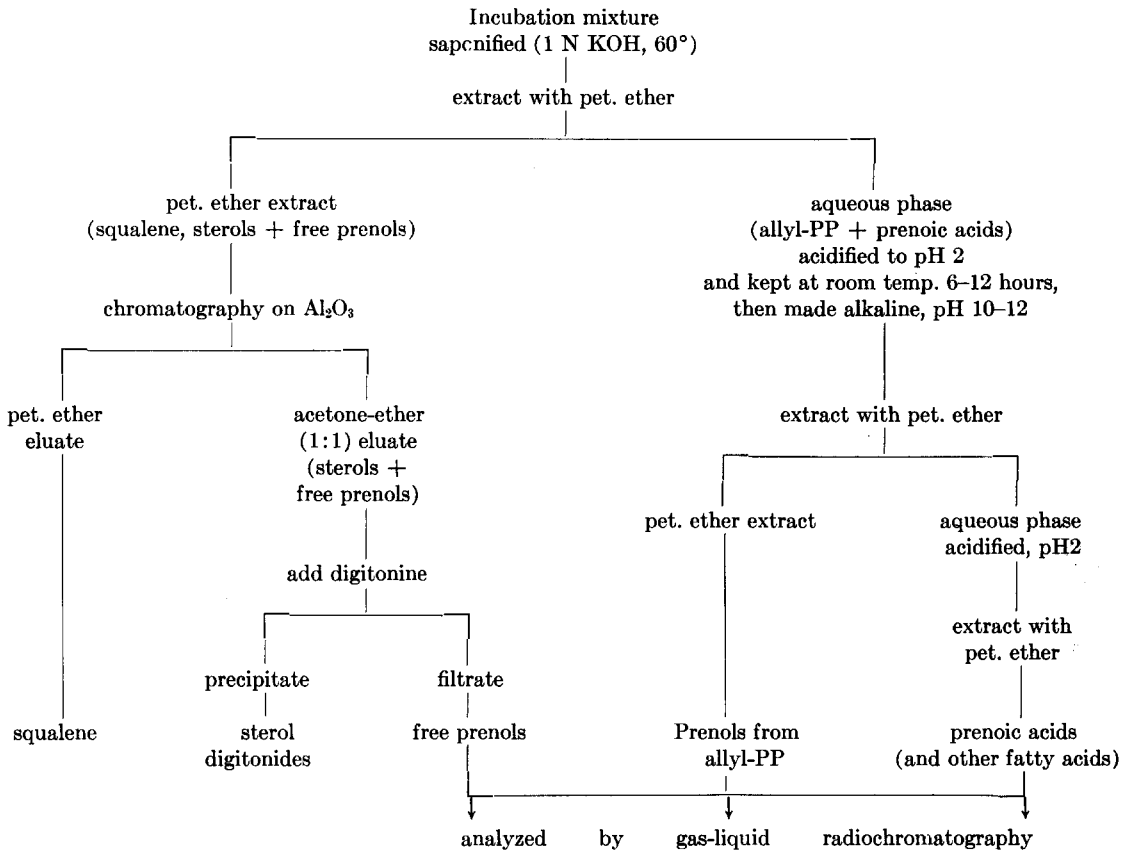
Isolation and Analysis of Incubation Products. The

[2] The following abbreviations are used: ATP, ADP, adenosine-5′-tri- and diphosphate; DPN, TPN, DPNH, TPNH, di-, triphosphopyridine nucleotide, oxidized and reduced; EDTA, ethylenediamine-tetraacetate; tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate; GSH, reduced glutathione; PCMB, *p*-chloromercuribenzoate; BAL, 2,3-dimercaptopropanol; allyl-PP, allyl pyrophosphates.

[3] The terms "prenol" and "prenoic acid" have been proposed by Popják and Cornforth (14) to denote alcohols and acids, respectively, which can be clearly dissected into isoprenoid units, —CH_2—$C(CH_3)$=CH—CH_2—. According to this nomenclature, which may be particularly useful in view of the increasing number of recognized long-chain polyisoprenoid substances, 3,3-dimethylallyl alcohol is denoted as monoprenol, geraniol as *trans*-diprenol, farnesol as *trans-trans*-triprenol, farnesoic acid as triprenoic acid, etc. The tertiary alcohols, linalool and nerolidol (isomers of geraniol and of farnesol) may be called *tert*-diprenol and *tert*-triprenol, respectively. Collectively they may be referred to as prenoid substances.

incubation mixtures were hydrolyzed at 60° for 1 hour after addition of 1 ml of 2 N KOH in 70% ethanol. The hydrolysis and the extraction of all enzymic products were carried out in the centrifuge tubes. In various experiments the following products were determined: squalene, cholesterol, allyl pyrophosphates, free prenols, and prenoic acids. All these products can be determined in one incubation. The methods used were essentially those described previously (9); the outline of the whole procedure is given in Chart 1.

CHART 1. FLOWSHEET OF PROCEDURES USED FOR ISOLATION OF ENZYMIC PRODUCTS BIOSYNTHESIZED FROM MEVALONATE-2-C^{14}



For purposes of gas-liquid chromatography, the acids were methylated with diazomethane. The analysis and identification of prenols and of prenoic acids by gas-liquid chromatography has been described (4, 14), and was made with the gas-density balance (15) or with the Argon detector (W. G. Pye & Co., Cambridge, England). Since the amounts of the biosynthetic C^{14}-prenols and C^{14}-prenoic acids were too small for detection by the gas-density balance, gas-liquid radiochromatography (4, 9) was the method employed, and a mixture of unlabeled prenols (dimethylallyl alcohol, linalool, geraniol, nerolidol, *cis-trans*-farnesol, and *trans-trans*-farnesol), in the ratios of 1/1/1/2/2/2 (v/v) or, as the occasion required, varying amounts of the methyl esters of unlabeled prenoic acids (3,3-dimethylacrylic, *cis*-geranoic, *trans*-geranoic, *cis-trans*-farnesoic, and *trans-trans*-farnesoic acids) were used as internal standards.

Spectrophotometric Studies. The dehydrogenation of prenols and of other alcohols by crystalline alcohol dehydrogenase from liver and yeast, and by dehydrogenases present in the F_{30}^{60}-enzyme preparations was measured by following the reduction of DPN at 340 mμ and at 37° in a recording Beckman-DK2 spectrophotometer equipped with a time drive and an electrically heated cell carrier. The reactions were started by the addition of the alcohol, the change in light extinction being recorded against a reference cuvette containing all ingredients except the alcohol.

In one set of experiments, substrate specificity for liver and yeast alcohol dehydrogenase was determined by measuring the initial rates of DPN reduction in the presence of various alcohols. Each cuvette contained 10 μmoles of of alcohol, 0.6 μmole of DPN, and 15 μg of

alcohol dehydrogenase in a final volume of 3 ml. In a second set, the equilibrium of DPN reduction was studied. Here each cuvette contained DPN (1.5 μ moles) in excess of the substrate alcohols (0.05–0.15 μmole), and 75 μg of alcohol dehydrogenase in a final volume of 3 ml.

The suspensions of crystalline alcohol dehydrogenases (from liver or from yeast) were dissolved on the experimental day in 2% bovine serum albumin. Among the various alcohols used, farnesol and geraniol have low solubility in water and therefore these alcohols were suspended in 0.1 M concentration in 2% bovine serum albumin solution; all other alcohols were added to the reaction mixture from a 0.1 M aqueous solution.

Sources of Enzymes, Coenzymes, and Other Substances. Crystalline horse liver alcohol dehydrogenase and crystalline yeast alcohol dehydrogenase were obtained from Boehringer & Söhne, Mannheim, Germany. The sources of all other biochemical reagents, enzymes, coenzymes, and of various chemicals were given in previous communications (9, 13). The origin and purity of specimens of prenols and of the methyl esters of prenoic acids has also been described (14). Dr. A. Eschenmoser of the Eidgenössische Technische Hochschule, Zurich, provided specimens of cyclized geranoic and cyclized farnesoic acid.

RESULTS

It has already been recorded (7, 9) that incubation of F_{30}^{60}-enzymes with DL-mevalonate-2-C^{14}, ATP, and Mg^{++} leads principally to the synthesis of allyl pyrophosphates with the simultaneous appearance of only small amounts (5% to 15% of the allyl pyrophosphates formed) of acidic substances. When, however, microsomes are added to an F_{30}^{60}-enzyme incubation in which the allyl pyrophosphates have already been synthesized during a preliminary incubation, a substantial part of the allylic derivatives is converted into carboxylic acids (Table 1). The Table also shows the appearance of C^{14} in free prenols, mainly in *trans-trans*-farnesol according to gas-liquid radiochromatographic analysis. This finding suggested that the radioactive acids were derived from the allyl pyrophosphates in two steps: dephosphorylation of the allyl pyrophosphates to free prenols, followed by dehydrogenation of the alcohols to acids. This assumption was borne out by the results of the experiment recorded in Table 2, in which a purified specimen of C^{14}-allyl pyrophosphates (9) was incubated with microsomes alone and with microsomes plus F_{30}^{60}-enzymes. The microsomes caused an almost complete hydrolysis of the allylic derivatives into free prenols; significant amounts of C^{14}-acids appeared only when F_{30}^{60}-enzymes were also added to the incubations. It was found, further, that the enzymes responsible for the formation of C^{14}-acids were almost entirely confined to a protein fraction of the soluble supernatant of rat liver homogenates precipitable with $(NH_4)_2SO_4$ between 30% and 60% saturation (Table 3).

These observations indicated, therefore, that the following sequence of reactions could account for the synthesis of C^{14}-acids from mevalonate-2-C^{14} in liver enzyme preparations: synthesis of allyl pyrophosphates, their subsequent hydrolysis by a microsomal phosphatase, and, finally, the dehydrogenation of the free prenols to acids by soluble enzymes. In the remainder of this paper detailed studies of the last two reactions are presented.

TABLE 1. EFFECT OF RAT LIVER MICROSOMES ON SYNTHESIS OF PRENOIC ACIDS FROM MEVALONATE-2-C^{14}*

Incubation	C^{14} Found in		
	Allyl-PP	Prenoic Acids†	Free Prenols
	cpm	*cpm*	*cpm*
Without microsomes	49,700	1,400	90
Microsomes added after 1 hour	17,600	36,400	3,615

* Each tube contained in a final volume of 5 ml, 1.0 mM DL-mevalonate-2-C^{14} (total of 177,000 cpm, i.e., 88,500 counts of the biologically active [+] mevalonate), 5 mM $MgCl_2$; 7.5 mM ATP; 10 mM NaF; 0.1 M phosphate buffer, pH 7.4, and F_{30}^{60}-enzymes (21 mg protein/ml). Incubations under N_2 for 2 hours at 37°. After 1 hour, microsomes (0.20 ml) were added to one of the tubes.

† Although this acidic fraction as isolated from the incubations contained also the various normal fatty acids, none of the radioactivity was associated with these, but only with prenoic acids. In every experiment the prenols as well as the acids were analyzed by gas-liquid radiochromatography.

TABLE 2. HYDROLYSIS OF C^{14}-ALLYL PYROPHOSPHATES BY RAT LIVER MICROSOMES AND EFFECT OF SOLUBLE ENZYMES ON PRENOLS*

Addition	C^{14} Found in		
	Residual Allyl PP	Free Prenols	Prenoic Acids
	cpm	*cpm*	*cpm*
None	340	5020	440
F_{30}^{60}-enzymes†	220	2900	2260

* Each tube contained in 1 ml, 5,500 cpm of C^{14}-allyl pyrophosphates (4,100 cpm, i.e., 0.053 μmole of farnesyl pyrophosphate), 0.05 ml microsomes, 5 mM $MgCl_2$, and 0.1 M tris-HCl buffer, pH 7.4. Incubation in air for 1 hour at 37°.

† 15 mg protein.

TABLE 3. PRODUCTION OF PRENOIC ACIDS FROM C^{14}-ALLYL PYROPHOSPHATES BY MICROSOMES AND SOLUBLE ENZYME FRACTIONS*

Soluble Enzymes Added		C^{14} Found in		
Fraction	Protein	Residual Allyl-PP	Free Prenols	Prenoic Acids
	mg	*cpm*	*cpm.*	*cpm*
F_0^{90}	13	520	1200	1500
F_0^{30}	13	300	3040	320
F_{30}^{60}	13	920	1000	1380
F_{60}^{90}	6.4	820	1880	360

* Each tube contained in 1 ml, 3,750 cpm of C^{14}-allyl pyrophosphates (2,800 cpm, i.e., 0.036 μmole of farnesyl pyrophosphate); 0.05 ml microsomes; 5 mM $MgCl_2$; 0.1 M phosphate buffer, pH 7.4; and fractions of a 105,000 × *g* supernatant of liver homogenate precipitated with $(NH_4)_2SO_4$ between various percentages of saturation. Incubation under N_2 for 1 hour at 37°.

Properties of the Microsomal Allyl Pyrophosphatase. Phosphate buffer was avoided in experiments designed to test the properties of the microsomal phosphatase since high concentrations (0.1 M) of phosphate were found to inhibit the enzyme.

For the determination of the pH optimum of this phosphatase, Na-citrate-HCl buffers (0.1 M, pH range 5.6 to 8.1) and tris-HCl buffers (0.1 M, range 6.9 to 9.0) were used. The optimum pH was around 7.0 (Fig. 1). The activity of the enzyme decreased fairly rapidly on the alkaline side, so that at pH 9.0 only 42% of the maximum activity could be demonstrated; on the acid side, however, there was some indication of a second peak of activity developing below pH 5.6. As the allyl pyrophosphates are unstable below pH 5.5 and cleave into inorganic pyrophosphate and prenols (9), measurements of enzymic hydrolysis at more acid pH values would be meaningless. The extent of the enzymic hydrolysis of the allyl pyrophosphates was measured by the amount of free prenols appearing, and not by release of phosphate; we have no data to show whether inorganic orthophosphate or pyrophosphate is liberated by the microsomal phosphatase.

Figure 2 illustrates the rate of hydrolysis of the allyl pyrophosphates by microsomes at pH 7.4; 50% of the substrate was hydrolyzed in 18 minutes, and 80% in 1 hour.

The microsomal allyl pyrophosphatase needed no Mg^{++} or other divalent metal ion for activation. The activity of the microsomes was not altered by dialysis for 5 hours against 0.02 M tris-HCl buffer, pH 7.4, containing 1 mM EDTA. Even when such dialyzed preparations were pretreated at 0° for 70 minutes with 5 mM EDTA, KCN, 8-hydroxyquinoline-5-sulfonic acid, or with 1,10-phenanthroline, the phosphatase activity was still the same as that of the untreated microsomes. In fact, with the exception of Mg^{++}, all the divalent ions tested (Ca^{++}, Ni^{++}, Co^{++}, Mn^{++}, Cu^{++}, and Zn^{++}, in order of increasing effectiveness) were inhibitory in a concentration of 5 mM (Table 4). A number of other substances, listed in Table 4, were also inhibitory, the most potent ones being NaF and, most surprisingly, crystalline bovine serum albumin. The F_{30}^{60}-enzymes containing 15 mg protein per ml, 2 mM glycine plus 10 mM glucose-6-phosphate, 0.1 M sodium pyrophosphate, 10 mM ATP, 2 mM dimethylallyl alcohol, 1 mM PCMB, and 1 mM N-ethyl maleimide had no effect on the hydrolysis of C^{14}-allyl pyrophosphates.

The extraction of microsomes with Lubrol-W (see under Methods) showed that the allyl pyrophosphatase was firmly attached to these particles. Although the treatment with the detergent dissolved about one-half of the microsomal proteins, nevertheless almost all the phosphatase activity remained in the Lubrol-insoluble particles.

When C^{14}-prenols (mostly C^{14}-farnesol, see under Methods) were incubated either with microsomes, or with microsomes plus F_0^{90}-enzymes, under conditions in which farnesyl pyrophosphate and mevalonate,

TABLE 4. INHIBITORS OF MICROSOMAL PHOSPHATASE*

Additions	Inhibition†
	per cent
$MgCl_2$, 5 mM	0
$CaCl_2$, 5 mM	14
$NiSO_4$, 5 mM	27
$CoCl_2$, 5 mM	56
$MnCl_2$, 5 mM	71
$Cu(NO_3)_2$, 5 mM	90
$ZnSO_4$, 5 mM	93
NaF, 5 and 10 mM	59
Potassium phosphate‡	17
Sodium arsenate‡	17
BSA,§ 0.4 mg/ml	54
BSA,§ 2.0 mg/ml	78
BSA,§ 4.0 mg/ml	81

* Each incubation contained in 1 ml, 2,500 cpm of C^{14}-allyl pyrophosphates (1,900 cpm, i.e., 0.024 μmole, in farnesyl pyrophosphate), 0.1 M tris-HCl buffer, pH 7.4, and 0.05 ml microsome suspension dialyzed against 1 mM EDTA in 0.02 M tris-HCl buffer, pH 7.4, for 5 hours at 5°. Additions and their concentrations were as shown. Aerobic incubations for 1 hour at 37°.

† The per cent inhibitions were calculated from a comparison of the hydrolysis of allyl pyrophosphates found in the absence of additions.

‡ pH 7.4, 0.1 M.

§ Bovine serum albumin.

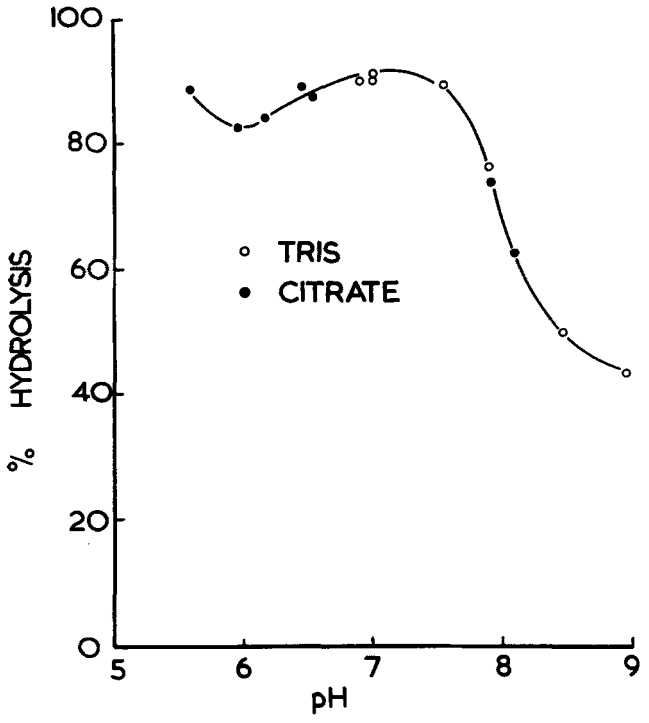


FIG. 1. Relation between pH and hydrolysis of allyl pyrophosphates by rat liver microsomes. Each tube contained in 1 ml, 2,500 cpm of C^{14}-allyl pyrophosphates (1,900 cpm, i.e., 0.024 μmole in farnesyl pyrophosphate); 5 mM $MgCl_2$, 0.1 M buffer, and 0.05 ml microsomes. Incubation under air at 37° for 1 hour. Extent of hydrolysis measured by appearance of free C^{14}-prenols.

respectively, are converted into squalene (9, 11), no synthesis of C^{14}-squalene or of C^{14}-allyl pyrophosphates could be demonstrated (Table 5). The free prenols are obviously not precursors of squalene, and the hydrolysis of the allyl pyrophosphates by microsomes is an irreversible process.

Conversion of C^{14}-Prenols into Acids by Liver Soluble Enzymes; Properties of the Enzymes. Free C^{14}-prenols were shown to be largely converted into acids in the presence of soluble enzymes (Table 5, incubation Nos. 3–5). The extent of the transformation was independent of the presence or absence of TPNH or of ATP. When the substrate was a prenol preparation consisting mostly of C^{14}-nerolidol, only about 20% of the total counts appeared in the acidic fraction (Table 5, incubation No. 6). This proportion of the counts corresponded to the primary C^{14}-prenol content of the substrate preparation. Clearly, only primary alcohols, as might be expected, could act as precursors of the acids.

It was shown in further experiments, which need not be detailed, that neither microsomes nor Mg^{++} were needed for the conversion of prenols into acids, but only the enzymes present in the F_{30}^{60}-protein fraction of the liver homogenates. Phosphate or tris-HCl buffers of the same pH were equally suitable for studying the transformation of the alcohols into acids.

As it appeared most probable that some dehydrogenase(s) were concerned in the conversion of the prenols into acids, it was somewhat intriguing that no definite need for a pyridine nucleotide could be demonstrated at first, in spite of the fact that the F_{30}^{60}-preparations were always dialyzed. As it was suspected that pyridine nucleotides were too firmly bound to the proteins to be removed by dialysis, a preparation of F_{30}^{60}-enzymes (5 ml; 39 mg protein/ml) was treated with about 1 g of Dowex-1 ion exchange resin for 15 minutes according to Tietz and Popják (16). This treatment depressed the conversion of C^{14}-prenols to acids by 30%, but the addition of DPN to the incubation completely overcame the depression and resulted in a 12% increase in the yield of acids as compared to that observed with untreated F_{30}^{60}-enzymes.

Figure 3 illustrates that the conversion of C^{14}-prenols into acids by a dialyzed F_{30}^{60}-preparation is fairly rapid, about 28% and 70% of the added substrate having been converted into acids in 2 and 15 minutes, respectively. The initial rate of this conversion could be increased to 42% in 2 minutes by the addition of DPN (0.33 μmole), and by ensuring rapid reoxidation of any DPNH formed by the use of pyruvate and lactic dehydrogenase.

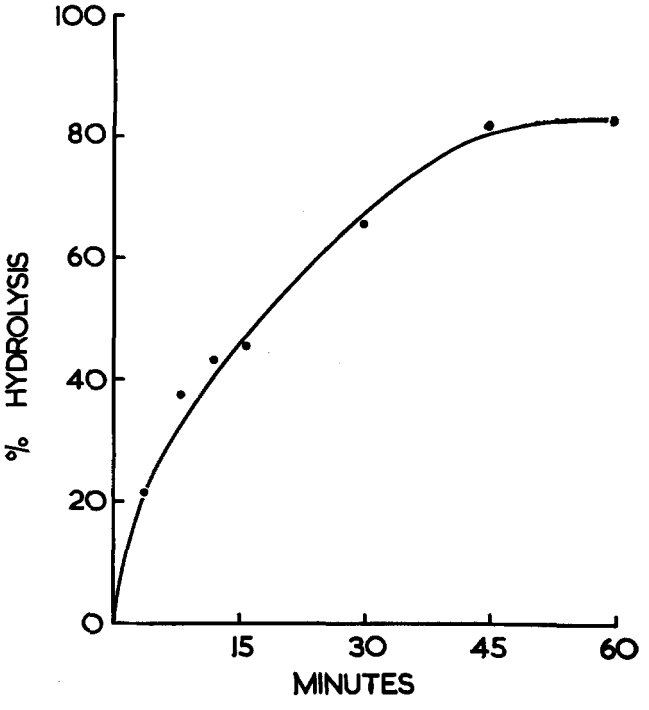


FIG. 2. Rate of hydrolysis of C^{14}-allyl pyrophosphates by rat liver microsomes at pH 7.4. Conditions of incubations as for Figure 1, except that tris-HCl buffer was used.

TABLE 5. FORMATION OF PRENOIC ACIDS FROM C^{14}-PRENOLS BY SOLUBLE ENZYMES OF RAT LIVER. FAILURE OF SYNTHESIS OF ALLYL PYROPHOSPHATES AND OF SQUALENE FROM PRENOLS*

Incubation No.	Additions	C^{14} Found in			
		Squalene	Free Prenols	Prenoic Acids	Allyl-PP
		cpm	*cpm*	*cpm*	*cpm*
1†	TPNH	1806	399	100	840
2‡	TPNH	0	5733	600	90
3‡	F_0^{90}, TPNH, ATP	0	347	6640	20
4‡	F_0^{90}, ATP	0	651	6900	30
5‡	F_0^{90}	0	777	6500	20
6§	F_0^{90}, TPNH, ATP	0	4158	1020	80

* Each tube contained in a final volume of 1 ml, 0.1 M K-phosphate buffer, pH 7.4; 5 mM $MgCl_2$; 30 mM nicotinamide; 10 mM NaF; and 0.05 ml of microsome suspension. Additions of F_0^{90}-enzymes (14 mg), of TPNH (1 μmole, generated with 2 μmoles of glucose-6-phosphate and glucose-6-phosphate dehydrogenase), and of ATP (7.5 μmoles) were made as shown. Incubations under N_2 for 1 hour at 37°.

† Substrate, C^{14}-allyl pyrophosphates: 3,750 cpm, of which 2,800 cpm (0.036 μmole) were in farnesyl pyrophosphate.

‡ Substrate, C^{14}-prenols: 7,500 cpm, of which 5,600 cpm (0.072 μmole) were in *trans-trans*-farnesol.

§ Substrate, C^{14}-prenols: 6,500 cpm, of which 4,200 cpm (0.054 μmole) were in nerolidol, and 1,300 cpm (0.017 μmole) in farnesol.

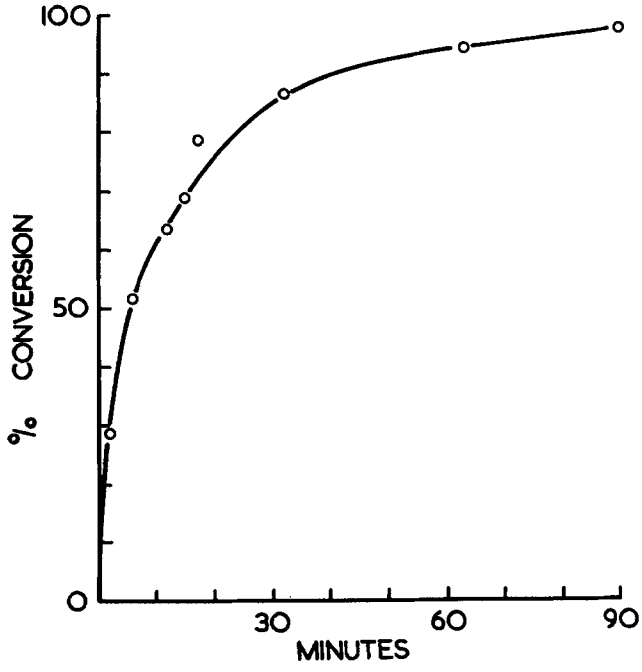


FIG. 3. Rate of conversion of C^{14}-prenols into acids by rat live soluble enzymes. Each tube contained in 1 ml, 2,200 cpm of C^{14}-prenols (1,900 cpm = 0.025 μmole of farnesol + geraniol); F_{30}^{60}-enzymes (15 mg protein); and 0.1 M phosphate buffer pH 7.4. Incubation under N_2 at 37°.

The enzymes responsible for the conversion of prenols into acids had a broad pH optimum lying between 7.2 and 8.2 (Fig. 4).

The dehydrogenation of C^{14}-prenols was almost completely inhibited (97%) by 1 mM PCMB. This inhibition was partially reversed (47%) in the presence of a small excess of GSH (1.5 mM). Other reagents for thiol groups, when tested in 1 mM concentration, were found to be inhibitory to a variable extent: N-ethyl maleimide gave a 40%, iodoacetamide a 22%, and iodoacetate only a 7% inhibition. BAL (2 mM) was also inhibitory (36%), and the inhibition caused by it was augmented to 72% by 1 mM sodium arsenite, which by itself was without effect. Silver and copper ions (0.5 mM) stopped completely the dehydrogenation of prenols. Much of the activity of F_{30}^{60}-preparations was lost on storage at −15° for more than 1 month, but it could be largely restored by the addition of 2 to 4 mM GSH.

Identification of the Prenoic Acids Derived from Mevalonate or from Prenols. The C^{14}-prenols and prenoic acids synthesized from mevalonate-2-C^{14} were identified by gas-liquid radiochromatography (4). Popják and Cornforth (14) have also reported on the behavior of the prenols and of the prenoic acids in gas-liquid chromatography, which allows the identification of these substances with confidence, provided the analyses are made both on columns with polar and nonpolar station-

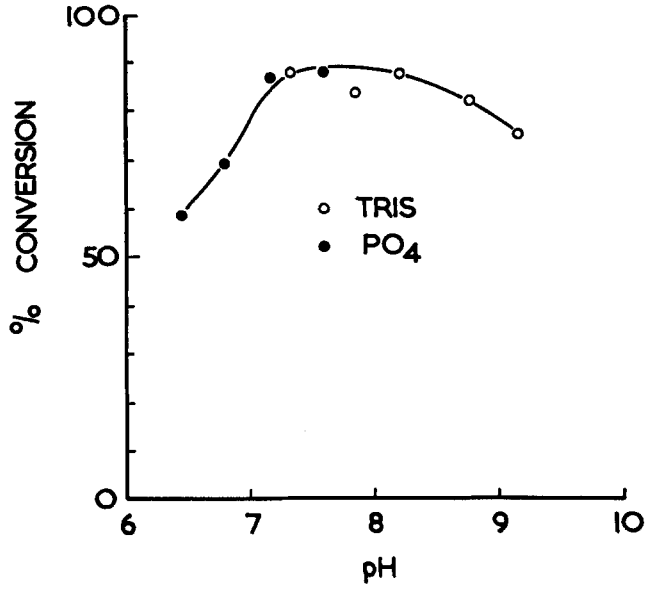


FIG. 4. Relation between pH and conversion of C^{14}-prenols into acids by rat liver soluble enzymes. Each tube contained in 1 ml, 2,500 cpm of C^{14}-prenols, of which 2,100 cpm were in farnesol and geraniol; F_{30}^{60}-enzymes (33 mg protein); and 0.1 M buffer. Incubation under air at 37° for 30 minutes.

ary phases. It was recognized in this laboratory some time ago (2, 3) that one of the main acids derived from mevalonate-2-C^{14} in liver homogenates was *trans-trans*-C^{14}-farnesoate (Fig. 6 *III*). The retention volume of the methyl ester of this acid was identical with that of the *trans-trans* component of synthetic methyl farnesoate. Furthermore, it was shown[4] that after catalytic hydrogenation of the above C^{14}-acid, it became inseparable by gas-liquid chromatography from an authentic specimen of methyl 3,7,11-trimethyldodecanoate, which is the saturated analogue of methyl farnesoate. Also, reduction of the methyl ester with $LiAlH_4$ in ether at −30° (14) gave *trans-trans*-C^{14}-farnesol.

Prenoic Acids with Retention Volumes Smaller than That of Farnesoate. In order to study these compounds more closely, a pooled specimen of C^{14}-prenoic acids was separated by gas-liquid chromatography at 197° into fractions emerging from the column before and after *trans-trans*-farnesoate. The fraction containing substances with the smaller retention volumes was then analyzed by gas-liquid radiochromatography at 100° on a column with ethylene glycol-adipic acid polyester stationary phase. By this procedure it was possible to compare more accurately the retention volumes of the radioactive methyl esters with those of internal (unlabeled) standards chromatographed simultaneously. We have identified in this way the methyl

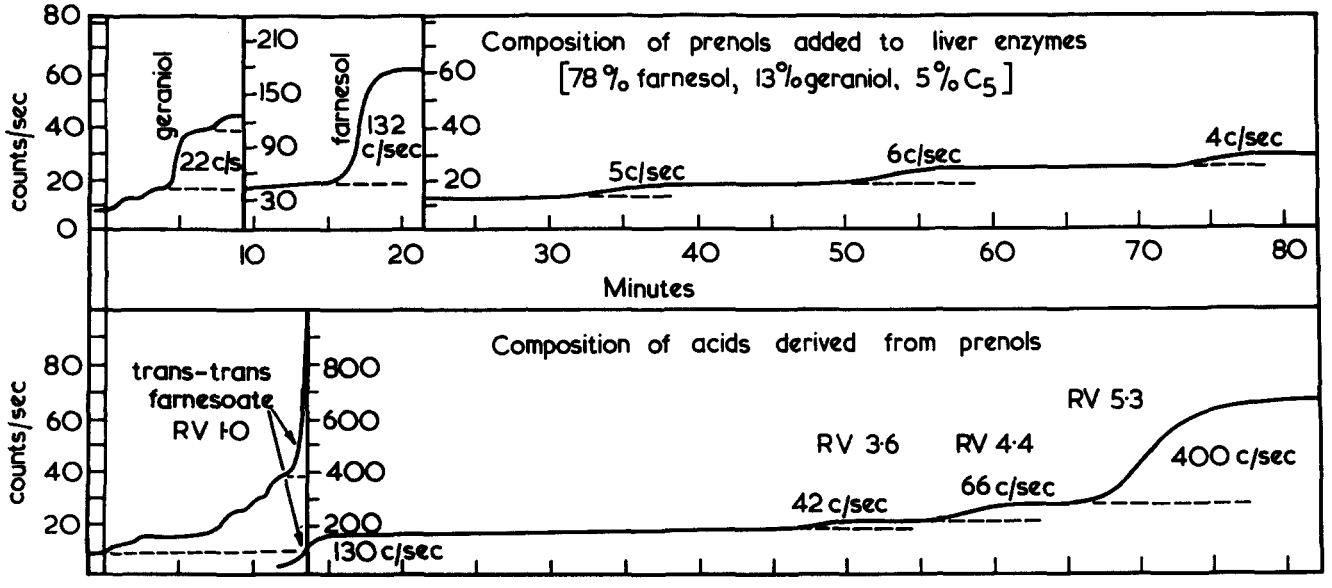


FIG. 5. Gas-liquid radiochromatographic analysis of C^{14}-prenols and acids derived from them. (*a*) C^{14}-prenols added to rat liver soluble enzymes; (*b*) C^{14}-acids derived from the C^{14}-prenols after 1-hour incubation at 37°. Four-foot column with ethylene glycol-adipic acid polyester stationary phase; 197°.

Gas-liquid radiochromatographic analysis of the C^{14}-acids derived from mevalonate-2-C^{14}, from C^{14}-allyl pyrophosphates, or from the primary C^{14}-prenols revealed identical components, and showed that in addition to farnesoate, other radioactive acids were also formed. Some of these had retention volumes smaller than that of farnesoate, and several emerged from the columns much later than farnesoate. Figure 5 is a typical record of the analysis of the C^{14}-methyl esters obtained by the incubation of C^{14}-prenols with F_{30}^{60}-enzymes. Table 6 summarizes the gas-liquid chromatographic analyses of the prenoic acids in eight experiments, and shows that no less than 13 acidic compounds were encountered.

[4] In collaboration with Mrs. M. Horning of the National Heart Institute, National Institutes of Health, Bethesda 14, Md.

esters of C^{14}-3,3-dimethylacrylate (Fig. 6 *I*), of C^{14}-*cis*-geranoate, and of C^{14}-*trans*-geranoate (Fig. 6 *II*).

Prenoic Acids with Retention Volumes Greater than That of Farnesoate. It was apparent from a balance of the counts in the acidic fractions and of the C^{14}-prenols used as substrates that the radioactive methyl esters with a retention volume greater than that of farnesoate could have been derived only from *trans-trans*-C^{14}-farnesol (Fig. 5). It was necessary to investigate whether these compounds occurred as natural enzymic products, or were artifacts produced during the extraction procedure. To check the latter possibility, a pool of the methyl esters of C^{14}-prenoic acids was subjected to gas-liquid chromatography, and the fraction corresponding to methyl *trans-trans*-C^{14}-farnesoate collected. This sample of 95% pure *trans-trans*-

C^{14}-farnesoate was then treated in the usual way, i.e., it was saponified with ethanolic potassium hydroxide, then acidified with HCl, extracted with petroleum ether, and methylated again. Radiochromatography showed that over 60% of the *trans-trans*-C^{14}-farnesoate had been transformed into a C^{14}-acid with a retention volume 5.78 times greater than that of the original compound. A similar phenomenon could be observed with a mixture of unlabeled *trans-trans* and *cis-trans*-farnesoate (Table 6). These results indicated that the extraction procedure was responsible for the conversion of farnesoate into compounds with a much larger retention volume. The nature of these compounds is not known but it is suspected that the one with retention volume 5.78 times that of farnesoate might be the monocyclic derivative (Fig. 6 *V*) of farnesoic acid (17, 18). It was also considered that these substances might be hydroxylated derivatives of farnesoic acid; however, attempted acetylation with acetic anhydride in pyridine did not change the retention volume of any of the radioactive fractions. Saponification, etc., of the methyl esters of *cis*-geranoic and *trans*-geranoic acids likewise resulted in a partial transformation of the acids into substances with retention volumes larger than those of the original methyl esters (Table 6). These esters had retention volumes very close to that of methyl *trans-trans*-farnesoate, and the substance derived from methyl *trans*-geranoate was indistinguishable in gas-liquid chromatography from a specimen of *β-cyclo*-geranoate (Fig. 6 *VI*).

TABLE 6. GAS-LIQUID CHROMATOGRAPHY OF METHYL C^{14}-PRENOATES*

Retention Volume of Prenoates		Fraction Identified as	Distribution of Radioactivity in Fractions Relative to *trans-trans*-Farnesoate
Relative to Methyl Stearate	Relative to *trans-trans*-Farnesoate		
0.02	0.03	3,3-Dimethylacrylate	0.07
0.09	0.15	*cis*-Geranoate	0.25
0.12	0.20	*trans*-Geranoate	0.31
0.32	0.54	Unidentified	0.85
0.45	0.78	*cis-trans*-Farnesoate	0.13†
0.54	0.94	*cis*-Geranoate derivative	—
0.59	1.00	*trans-trans*-Farnesoate	1.00
0.65	1.12	*β-cyclo*-Geranoate	—
0.80	1.36	Unidentified	0.14†
1.30	2.21	Unidentified	0.20†
2.20	3.74	Unidentified	0.07
2.76	4.78	*cis-trans*-Farnesoate derivative	0.69
3.34	5.78	*trans-trans*-Farnesoate derivative	1.91
3.60	6.12	Unidentified	0.24†
4.90	8.33	Bi-*cyclo*-farnesoate	0.54†

* Derived from C^{14}-prenols, or from mevalonate-2-C^{14}. Gas-liquid chromatography on ethylene glycol-adipic acid polyester columns at 197°. Mean values derived from eight experiments.
† Fractions not always present.

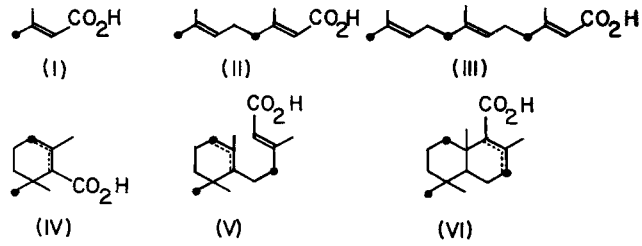


FIG. 6. Prenoic acids biosynthesized from mevalonate-2-C^{14} (*I–III*), and cyclized forms of geranoic and farnesoic acids (*IV–VI*). Positions marked with closed circles are those derived from C-2 of mevalonate.

Table 6 shows that the origin of some acids is still unidentified, but we are inclined to believe that the principal compounds, formed enzymically, are the mono-, di-, and triprenoic acids, and that most of the others are probably derived from the latter two by cyclization and isomerization during the isolation procedures.

As a further check on the origin of the C^{14}-acids, derived ultimately from mevalonate-2-C^{14}, we have studied the conversion of unlabeled geraniol and farnesol into acids by the F_{30}^{60}-enzymes. The specimen of geraniol was 98% pure and contained only 2% nerol. The preparation of "farnesol" contained 56% *trans-trans*-farnesol, 32% *cis-trans*-farnesol, and 12% nerolidol. As our aim was to obtain maximum yields of acids for gas-liquid chromatographic analysis, the DPNH generated during prenol dehydrogenation (see below) was oxidized continuously to DPN by the addition of the substrates (ADP and PEP) for the pyruvic kinase-lactic dehydrogenase system, which is present in abundance in our F_{30}^{60}-preparations. Two incubations of 10 ml each were set up containing the following: 0.1 M K-phosphate buffer, pH 7.4; F_{30}^{60}-enzymes, 19 mg protein per ml; DPN, 0.5 mM; ADP, 1.5 mM; PEP, 1.3 mM; $MgCl_2$, 5 mM; and geraniol, 1.4 mM in one experiment, and "farnesol," 2 mM in the other. The incubations at 37° lasted for 85 minutes. In the first experiment practically all the geraniol was converted into *trans*-geranoic acid and into the substance thought to be β-cyclogeranoate (Fig. 6 *IV*), both having been identified by gas-liquid chromatography.

In the experiment with "farnesol" only 17% of the total amount of substrate was converted into acid, but

all of this was derived entirely from the *trans-trans*-farnesol component of the substrate; the *cis-trans*-farnesol and nerolidol were recovered unchanged. Gas-liquid chromatography of the methyl esters of the acids formed showed these to consist of *trans-trans*-farnesoate and of a substance with a retention volume of 5.78 times that of methyl *trans-trans*-farnesoate.

The Role of Liver Alcohol Dehydrogenase and Aldehyde Dehydrogenase in the Origin of Prenoic Acids. It seemed most probable to us that liver alcohol dehydrogenase was involved in the transformation of the prenols into acids. In order to test this idea it was necessary to ascertain first whether the prenols could act as substrates for alcohol dehydrogenase, as no information was available on the subject. Table 7 shows that the rates

TABLE 7. SUBSTRATE SPECIFICITY OF HORSE LIVER ALCOHOL DEHYDROGENASE *

Alcohol	Initial Rate† ΔE_{340}/10 Minutes
3-Methylbut-3-en-1-ol (isopentenol)	0.44
3,3-Dimethylallyl alcohol	0.29
Geraniol	0.40
Nerol (*cis*-geraniol)	0.19
trans-trans-Farnesol	0.21
cis-trans-Farnesol	0.15
Potassium mevalonate	0.00
Allyl alcohol	0.58
isoAmyl alcohol	0.24
Pentan-2-ol	0.05
Ethanol	0.37

* Each cuvette contained in 3 ml, 0.1 M NaOH-glycine buffer, pH 9.5; crystalline horse liver alcohol dehydrogenase, 15 μg; 0.2 mM DPN; and 3.33 mM alcohol. The reaction was started by the addition of the alcohol, and the course of DPN reduction followed at 340 mμ and at 37°, using cuvettes with 1-cm light path.

† The initial rate of DPN reduction was calculated from the extrapolation of the linear part of the light-extinction curve to 10 minutes. A change of 0.1 in the value of E_{340} is equivalent to the reduction of 0.048 μmole of DPN under our conditions.

of dehydrogenation of isopentenol, dimethylallyl alcohol, geraniol, and farnesol by liver alcohol dehydrogenase compare favorably with that of ethanol. Nerol (*cis*-geraniol) and *cis-trans*-farnesol were dehydrogenated at a slower rate than their *trans*-isomers. The rate of dehydrogenation of a few other alcohols is also shown in Table 7. As was to be expected, the tertiary alcohols, *d*-linalool and nerolidol, were completely unreactive. DL-Mevalonate (as the potassium salt) was also inert toward the alcohol dehydrogenase. Yeast alcohol dehydrogenase was tested for specificity of substrates under the same conditions as the horse liver enzyme, and although it showed the expected activity toward ethanol (initial rate of dehydrogenation gave a ΔE_{340}/10 minutes of 0.58), it was completely unreactive with isopentenol, dimethylallyl alcohol, geraniol, and farnesol.

As is well known, the rate of dehydrogenation of ethanol with liver alcohol dehydrogenase is faster at pH 9.5 than at pH 7.4. The initial rate of the dehydrogenation of geraniol was the same at these two pH values, while with dimethylallyl alcohol the initial rate of dehydrogenation was faster at pH 7.4 than at pH 9.5 (ΔE_{340}/10 minutes = 0.47 at pH 7.4, against ΔE_{340}/10 minutes = 0.29 at pH 9.5).

In experiments designed to study the equilibria of the dehydrogenase reaction (see under Methods), it was found that at pH 7.4 the reduction of DPN in the presence of ethanol was barely detectable, a fact that was not unexpected since equilibrium is known to be greatly in favor of aldehyde reduction (19). In contrast, dimethylallyl alcohol and geraniol were quite efficiently dehydrogenated (Fig. 7*b*). Assuming that the amount of alcohol dehydrogenated was equal to the amount of DPN reduced, the values for the equilibrium constant at 37°, at pH 7.4

$$\left[K_{eq} = \frac{(\text{aldehyde})\ (\text{DPNH})\ (H^+)}{(\text{alcohol})\ (\text{DPN})}\right]$$

were 1.1×10^{-12} for ethanol; 2.8×10^{-10} for dimethylallyl alcohol, and 4.4×10^{-11} for geraniol.

Spectrophotometric experiments, similar to those described for crystalline liver alcohol dehydrogenase, were also done with the F_{30}^{60}-enzymes and the various alcohols. The F_{30}^{60}-enzymes were very active in reducing DPN (but not TPN) in the presence of prenols, and the initial rate of DPN reduction was proportional to enzyme concentration in the range studied (0.5 to 4 mg protein/ml). When experiments similar to those illustrated in Figure 7*b* were reproduced with F_{30}^{60}-enzymes (1.5 mg/ml), it was found that, as with crystalline alcohol dehydrogenase, both the initial rate and the extent of the dehydrogenation of dimethylallyl alcohol and of geraniol were greater than those of ethanol (Fig. 7*a*). However, for each alcohol tested (including ethanol), more DPN was reduced than in the corresponding experiment with crystalline alcohol dehydrogenase alone. The different levels of the equilibrium attained during dehydrogenation of prenols by crystalline liver alcohol dehydrogenase and by the F_{30}^{60}-enzymes are clearly seen by comparing Figures 7*a* and 7*b*. An accurate determination of the amount of DPN produced by the alcohols with F_{30}^{60}-enzymes was difficult because of a slow reoxidation of the DPNH generated. This phenomenon was more

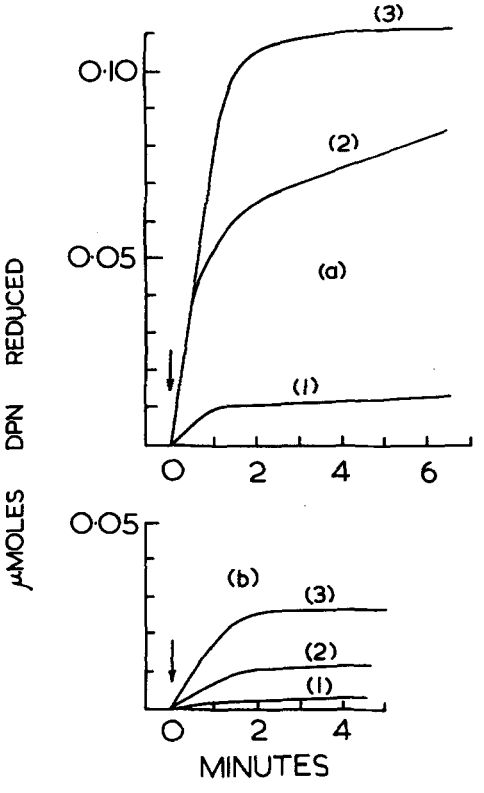


FIG. 7. Dehydrogenation of alcohols by horse liver alcohol dehydrogenase and by rat liver soluble enzymes. The curves represent the formation of DPNH as a function of time after the addition of 0.1 µmole of alcohol. Each cuvette contained in 3 ml, 0.5 mM DPN, 0.1 M phosphate buffer pH 7.4, and liver alcohol dehydrogenase (25 µg/ml) in (*b*); or F_{30}^{60}-enzymes (1.5 mg protein/ml) in (*a*). Curves (*1*): reaction with ethanol; curves (*2*): reaction with geraniol; and curves (*3*): reaction with dimethylallyl alcohol.

pronounced at pH 9.5 than at pH 7.5, and was due to a DPNH-oxidase present in the F_{30}^{60}-enzymes. In one instance the course of DPN reduction in the presence of dimethylallyl alcohol was corrected for this DPNH oxidation by observing the rate of oxidation of DPNH added to a control cuvette. It was calculated that 0.16 µmole of DPN was reduced by 0.1 µmole of dimethylallyl alcohol.

Since it is reasonable to assume that the equilibrium position of prenol dehydrogenated by horse liver alcohol dehydrogenase is similar to that catalyzed by rat liver alcohol dehydrogenase, the reduction of nearly 2 moles of DPN per mole of prenol in the F_{30}^{60}-preparations strongly suggests the participation of a second dehydrogenase. We suggest that this second enzyme is an aldehyde dehydrogenase which catalyzes the irreversible dehydrogenation to acids of the aldehydes generated from the prenols by liver alcohol dehydrogenase. Certainly, we could not demonstrate a reduction of 3,3-dimethylacrylate with DPNH and F_{30}^{60}-enzymes. On the other hand, when unlabeled farnesal (73% *trans-trans* + 27% *cis-trans* isomer, 1.2 µmoles) was incubated at pH 7.4 with F_{30}^{60}-enzymes (100 mg in 4 ml) for 1 hour at 37°, the *trans-trans*-isomer (but not the *cis-trans*-isomer) was converted into *trans-trans*-farnesoic acid, which was identified by gas-liquid chromatography.[5]

Synthesis of Allyl Pyrophosphates, Prenols, and Prenoic Acids in Vivo. In order to decide whether the prenoic acids found under the highly artificial conditions of the *in vitro* experiments had any physiological significance, we gave 5.4 µmoles (2.5×10^6 cpm) of DL-mevalonate-2-C^{14} by stomach tube to a 22-g mouse. The animal was killed 70 minutes later, and the liver analyzed for allyl pyrophosphates, free prenols, prenoic acids, squalene, and for cholesterol, by the same procedures as described for the enzyme incubations. Of the injected dose of DL-mevalonate, 2.35% (or 4.7% of the biologically active [+] isomer) was found incorporated into various components of the liver. Expressed as a percentage of total counts found in the liver, 11% was found in sterol digitonides, 46% in squalene, 4% in allyl pyrophosphates, 16% in free prenols, and 23% in prenoic acids. Upon gas-liquid radiochromatography, the main component of the prenoic acids was identified as *trans-trans*-farnesoate; *trans-trans*-farnesol was the main constituent of the prenols.

DISCUSSION

Evidence has been presented that in a liver enzyme system, mevalonate is a precursor not only of squalene but also of certain prenoic acids. These acids are formed by a mechanism completely different from that of the synthesis of normal fatty acids. This mechanism consists of the dephosphorylation of allyl pyrophosphates (intermediates in squalene biosynthesis) and subsequent dehydrogenation of the resulting prenols by liver alcohol dehydrogenase and aldehyde dehydrogenase into the corresponding acids. The reason why the synthesis of prenoic acids has not been reported from laboratories studying squalene biosynthesis with yeast enzymes is most probably due to the fact that yeast alcohol dehydrogenase is totally inactive toward prenols. A similar observation had already been made by Bliss (20) with vitamin A.

The microsomal phosphatase, which hydrolyzes the allyl pyrophosphates, has properties somewhat

[5] The retention volume of *trans-trans*-farnesal is very close to that of *trans-trans*-farnesol on both ethylene glycol-adipic acid polyester columns and on Apiezon-L columns at 197°, but is widely different from the retention volume of methyl *trans-trans*-farnesoate.

different from other phosphatases; its pH optimum is quite different from that of serum alkaline phosphatase, but is close to those of three other phosphatases present in microsomes: glucose-6-phosphatase (21), 5′-nucleotidase (22), and phosphatidic-acid phosphatase (23). Whether or not the phosphatase activity of the microsomes studied by us is specific for the allyl pyrophosphates cannot be decided, as these compounds are hydrolyzed by a variety of phosphatases, such as intestinal alkaline phosphatase, prostatic acid phosphatase, and snake venom phosphatases (9). Contrary to the needs of some phosphatases (e.g., alkaline phosphatase or snake venom phosphatases), the microsomal enzyme needs no Mg^{++} for activation, and chelating agents or sulfhydryl inhibitors had no effect on it. The inhibition of the microsomal phosphatase by bovine serum albumin was surprising; the mechanism of action of the albumin is probably indirect and may be attributed to a binding of the allyl pyrophosphates to this protein. It was found in experiments, not recorded here, that potassium farnesoate and the mono-, di-, and triprenols, in concentrations of 0.4 to 2 mM, could partially reverse the inhibition caused by albumin. Possibly these substances and the allyl pyrophosphates were competing for identical binding sites on the albumin. It is of interest to point out that the microsomes also contain the squalene synthetase system (9) which converts two molecules of farnesyl pyrophosphate into squalene, but only in the presence of a reduced pyridine nucleotide, TPNH or DPNH. In the absence of these coenzymes the squalene synthetase system is completely ineffective, and the action of the microsomal phosphatase comes to the fore, destroying the substrate for the squalene synthetase, as free prenols cannot be utilized for the synthesis of squalene.

The experimental evidence presented on the dehydrogenation of prenols to acids indicates that this occurs in two steps, with aldehydes as intermediates, involving both an alcohol dehydrogenase and an aldehyde dehydrogenase. We have not examined the possibility that the acids may arise from the prenols by the action of the liver alcohol dehydrogenase alone, through a dismutation of the aldehydes to acid and alcohol (24). It does not appear, however, to be a very likely mechanism for the formation of prenoic acids, since spectrophotometric studies with the F_{30}^{60}-enzymes showed the reduction of nearly 2 moles of DPN by 1 mole of prenol.

It is well known that both the alcohol and aldehyde dehydrogenases of liver contain SH-groups at their active sites (25). The inhibition of the dehydrogenases present in our F_{30}^{60}-preparations by PCMB, N-ethylmaleimide, iodoacetamide, Ag^{+}, and Cu^{++} suggests very strongly that these two dehydrogenases are responsible for the formation of the prenoic acids. The failure of iodoacetate to inhibit the conversion of prenols to acids by the F_{30}^{60}-preparations is in harmony with the observation that liver alcohol dehydrogenase is insensitive to this reagent (26). The sensitivity of the F_{30}^{60}-dehydrogenases to PCMB was such that PCMB was used in preparative incubations when C^{14}-prenols were being made from C^{14}-allyl pyrophosphates in the presence of both microsomes and F_{30}^{60}-enzymes (see under Methods). The use of PCMB in such incubations depended also on the fact that the microsomal phosphatase was completely insensitive to this reagent.

Our finding, that the enzymes converting prenols into acids are confined largely to the F_{30}^{60}-fraction of the soluble supernatant of liver homogenates, is also consistent with the properties of liver alcohol and aldehyde dehydrogenases, which are known to be precipitated with $(NH_4)_2SO_4$ between 55% and 75%, and 51% and 60% saturation, respectively (25, 27).

Spectrophotometric experiments with the F_{30}^{60}-enzymes showed clearly that the dehydrogenation of prenols was a DPN-linked reaction. The observation that the C^{14}-prenols could be converted into acids by the F_{30}^{60}-enzymes, even in the absence of added DPN, can be readily explained by the assumption that the enzyme preparation contained an amount of bound DPN sufficient for the dehydrogenation of the small quantities of C^{14}-prenols (0.01 to 0.03 μmole) used in the experiments. The DPN-oxidase present in the F_{30}^{60}-preparations would ensure, moreover, that the DPNH generated is reoxidized to DPN. Hence, for the dehydrogenation of 0.01 to 0.03 μmole of prenols, even catalytic amounts of DPN would suffice. The firm binding of DPN to the apo-enzyme in alcohol dehydrogenase is well known. The above assumption is supported by the finding that treatment of the F_{30}^{60}-preparation with Dowex-1 ion exchange resin caused some inactivation, which could be fully compensated by the addition of DPN.

The irreversible DPN-linked dehydrogenation of prenols to acids by F_{30}^{60}-enzymes is also in harmony with our assumption that aldehyde, as well as alcohol dehydrogenase, is involved in the process. Racker (28) observed that acetate could not be reduced to acetaldehyde with DPNH and liver aldehyde dehydrogenase. Thus the quantitative conversion of prenols to acids, which we have observed, could be explained only by the participation of two enzymes, since the equilibrium of the reaction with prenols catalyzed by alcohol dehydrogenase alone was always in favor of the alcohol.

The presence of aldehyde dehydrogenase, on the other hand, would drive the reaction toward complete dehydrogenation.

The finding that the prenols are highly specific substrates for liver (but not yeast) alcohol dehydrogenase suggests to us that the physiological role of this enzyme is connected primarily with the metabolism of intermediates of sterol biosynthesis and of similar natural products, and only fortuitously with dealing with man's social habit of consuming ethanol.

Evidence has been accumulated that the prenoic acids are 3,3-dimethylacrylic, *trans*-geranoic, and *trans-trans*-farnesoic acids, together with artifacts produced from these during extraction procedures. We have had no evidence for the presence of hydroxy acids. The dehydrogenases converting the prenols into acids appear to be specific for the *trans*-isomers of alcohols, as no formation of *cis-trans*-farnesoic acid from *cis-trans*-farnesol could be observed. The small amounts of the *cis*-isomers of C^{14}-geranoic acid, and of *cis-trans*-C^{14}-farnesoic acid derived from the C^{14}-prenols, are believed to arise through isomerization during the extraction procedure. The transformation of both geranoic and farnesoic acids into what we believe to be their cyclized forms under the relatively mild conditions of our extraction procedure appears somewhat surprising, since the acid catalyzed cyclization of these substances usually requires much more vigorous conditions (17, 18).

In some earlier communications from this laboratory (2, 3) it was stated that radioactive "terpenoid" acids were formed in liver homogenates, not only from mevalonate-2-C^{14} but also from mevalonate-1-C^{14}. This was a genuine observation in the sense that the particular batch of mevalonate-1-C^{14} gave rise to C^{14}-prenoic acids. One of us (Popják) repeated these earlier experiments with a new batch of mevalonate-1-C^{14} and failed to find an incorporation of C^{14} into prenoic acids: the samples contained only a few insignificant counts. It is suspected that the original batch of mevalonate-1-C^{14} may have contained label on some position other than the carboxyl carbon.

Synthesis of prenoic acids from mevalonate is not restricted to the highly artificial conditions of incubations of liver homogenate or of its fractions. Unpublished observations in this Unit by K. Fletcher and N. B. Myant showed that the same prenoic acids as those found in homogenates occurred in liver slices metabolizing mevalonate. Elwood *et al.* (29) reported recently that for every 22 atoms of C^{14} incorporated into cholesterol from mevalonate-2-C^{14} by liver slices, 1 atom of C^{14} appeared in the fatty acid fraction. Garattini *et al.* (30) have found that 2 hours after the intraperitoneal injection of mevalonate-2-C^{14} into 12-day-old rats, the radioactivity recovered in liver fatty acids was as great as that recovered in liver cholesterol, but the radioactive acids have not been identified. Our experiment with the mouse showed that the radioactivity found in liver fatty acids was associated mainly with *trans-trans*-farnesoate.

The physiological role of the prenoic acids is at present a matter of conjecture. Clearly, they represent an alternative pathway for the metabolism of the allyl pyrophosphates, and consequently may be involved in the regulation of cholesterol synthesis in two ways. The effect of the hydrolysis of the allyl pyrophosphates to free prenols by itself is to decrease the amount of substrate available for sterol synthesis. A further possibility is that the prenoic acids also exert an inhibitory action on various enzymic reactions of cholesterol biosynthesis. Wright and Cleland (31) were the first to observe an inhibition of cholesterol biosynthesis from mevalonate by farnesoic acid in liver homogenates, and this was confirmed in our laboratory. It was also shown by Levy and Popják (13) that farnesoate inhibited mevalonic kinase, and by Popják *et al.* (32) that some analogues of farnesoic acid were quite powerful inhibitors of cholesterol synthesis from mevalonate *in vitro*. In spite of these highly suggestive results it remains for future work to determine precisely the function and metabolism of prenoic acids *in vivo*.

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