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Studies on the biosynthesis of cholesterol: XII. synthesis of allyl pyrophosphates from mevalonate and their conversion into squalene with liver enzymes*

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SUMMARY

The biosynthesis of allyl pyrophosphates (the pyrophosphates of dimethylallyl alcohol, geraniol, and of farnesol) from DL-mevalonate-2-C^{14} and from (-)5-phosphomevalonate-2-C^{14} with soluble liver enzymes in the presence of ATP and Mg^{++} is described. The allyl pyrophosphates were partially purified and their properties studied. They are unstable below pH 5 and cleave into inorganic pyrophosphate (identified by the use of purified yeast inorganic pyrophosphatase) and allylic alcohols. The alcohol components were identified by gas-liquid radiochromatography after hydrolysis of the pyrophosphates by prostatic, intestinal, microsomal, and snake venom phosphatases. Farnesyl pyrophosphate, which was the principal product among the allyl pyrophosphates, was shown to be the precursor of squalene in the liver enzyme system just as was reported for yeast. The enzyme or enzymes responsible for the conversion of farnesyl pyrophosphate into squalene are attached to microsomal particles and need either TPNH or DPNH and a divalent cation (Mg^{++}, Mn^{++}, or Co^{++}) as cofactors. Slightly better yields of squalene were obtained with TPNH than with DPNH. The squalene synthesizing system was strongly inhibited by p-chloromercuribenzoate and N-ethyl maleimide, but was not affected by iodoacetamide; the optimal pH was 7.4. Addition of soluble enzymes to this system reduced the yield of squalene and caused some conversion of the allyl pyrophosphates into carboxylic acids.

Recent 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), 5-diphosphomevalonate and 3-methylbut-3-en-1-yl pyrophosphate (isopentenyl pyrophosphate) have been recognized as successive intermediates in the synthesis of squalene (2 to 5). Furthermore, Lynen *et al.* (4) demonstrated that a protein fraction of yeast extracts catalyzed the synthesis from mevalonate of farnesyl pyrophosphate which was converted into squalene on addition of yeast cell particles and reduced pyridine nucleotides. More recently it was shown that 3,3-dimethylallyl pyrophosphate and geranyl pyrophosphate are intermediates preceding the formation of farnesyl pyrophosphate (6).

Experiments with liver enzymes have indicated 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 to 10). In the present paper we report the synthesis, from mevalonate-2-C^{14} and from (-)5-phosphomevalonate-2-C^{14}, of acid-labile hydrophilic derivatives of dimethylallyl alcohol, geraniol, and of farnesol with soluble liver enzymes. These substances have been identified as pyrophosphate esters and some of their properties defined. Detailed studies on the synthesis of squalene from farnesyl pyrophosphate are also described. Brief accounts of these experiments have already appeared (11, 12).

* The following abbreviations are used: DPN and DPNH, diphosphopyridine nucleotide and its reduced form; TPN and TPNH, triphosphopyridine nucleotide and its reduced form; G-6-P, glucose 6-phosphate; ATP, adenosine triphosphate; EDTA, ethylene diamine tetraacetate; BAL, 2,3-dimercaptopropanol; GSH, glutathione; PCMB, p-chloromercuribenzoate.

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METHODS AND MATERIALS

Enzyme Preparations. Rat liver homogenates were made, as described in detail previously (13), in 0.1 M potassium phosphate buffer, pH 7.4 containing 5 mM $MgCl_2$ and 30 mM nicotinamide following the method of Bucher and McGarrahan (14). The preparation of microsomal and soluble enzyme fractions from the homogenates has also been described (15). After sedimentation of the microsomes at 105,000 $\times$ *g*, these cell particles were washed by suspension in buffer from which nicotinamide had been omitted (0.1 M potassium hydrogen phosphate buffer, pH 7.4, with 5 mM $MgCl_2$). After resedimentation at 105,000 $\times$ *g* for 45 minutes, the washed microsomes were suspended in a volume of the same buffer equal to one-tenth the volume of the liver homogenate from which they had been prepared. Nicotinamide was omitted during washing of microsomes in order to ensure destruction of pyridine nucleotides remaining attached to the particles.

The soluble supernatant of the rat liver homogenate was fractionated by the addition of solid ammonium sulfate. The active enzyme preparation used was that protein fraction which precipitated between 30 and 60 per cent ammonium sulfate saturation (F_{30}^{30}-enzymes). The precipitate was collected by centrifuging at 10,000 $\times$ *g* for 20 minutes; it was dissolved in 0.02 M $KHCO_3$ and dialyzed against the same for 3 to 4 hours. After clarifying the dialyzed solution by centrifugation, its protein content was 30 to 50 mg. per ml.

Incubations. These were made at 37°C, usually for 1 or 2 hours. One ml. anaerobic incubations were made in small ground-glass stoppered tubes, which were flushed with N_2 before and after filling and then securely stoppered. Larger anaerobic incubations were made under a continuous stream of N_2.

Extraction and Separation of Incubation Products. Assay of Squalene. The incubations were usually terminated by the addition of an equal volume of 15 per cent KOH (w/v) in 85 per cent ethanol. When the synthesis of squalene was measured, 10 mg. of carrier squalene was also added, and the mixture saponified by heating at 70°C for 1 hour. The unsaponifiable compounds were extracted with petroleum ether (b.p. 40°-60°C) and the squalene separated by chromatography on alumina washed with methylformate (16), squalene being eluted with petroleum ether (17). In one experiment squalene was further identified through the thiourea adduct. To the chromatographed specimen (after evaporation of the solvent) a few milliliters of a saturated methanolic solution of thiourea and a few milligrams of finely powdered thiourea were added. After thorough shaking the mixture was left at room temperature for a few days. The crystals of the clathrate were decomposed with water and the squalene extracted with petroleum ether.

Assay of Allyl Pyrophosphates and Carboxylic Acids. The simplest assay of the allyl pyrophosphates synthesized from C^{14}-labeled precursors was based on their stability toward alkaline hydrolysis and their instability in the presence of acid (see Results) as they cleave completely at pH 1 to 2 into inorganic pyrophosphate and alcohols, the latter being readily extractable with petroleum ether. After extraction of unsaponifiable substances from the saponified incubations (see preceding paragraph), 2 mg. of a mixture of dimethylallyl alcohol, geraniol, nerolidol, and of farnesol was added and the pH of the digest adjusted to 1 to 2 with HCl (or H_2SO_4). After one to several hours the mixture was made alkaline (pH 10 or above) with aqueous KOH and the free alcohols extracted with petroleum ether. Carboxylic acids were finally extracted by acidifying again the aqueous phase and extracting the acids with petroleum ether.

Assay of C^{14}. This was done with a thin mica-window Geiger-Müller counter (window thickness 1.2 mg. per $cm.^2$), with an efficiency of about 6 per cent. A given petroleum ether extract was first evaporated to a measured volume and an aliquot of this (usually one-tenth) plated on a plastic planchette of 2 $cm.^2$ area lined with lens paper. All counts were made at negligible thickness (less than 1 mg. per $cm.^2$). With samples of free alcohols the planchettes were counted immediately upon evaporation of the petroleum ether at room temperature, so as to minimize loss of counts because of the volatility of these alcohols. In the presence of carrier alcohols the counting rate of a given planchette diminished by less than 5 per cent in the first 20 minutes; experiments with known solutions have demonstrated that quantitative analyses can be achieved by this technique.

Analysis of Alcohols and Acids. Gas-liquid radiochromatography (18) was the method employed for the identification of both alcohols and acids produced from mevalonate-2-C^{14}. Acids were methylated with diazomethane before analysis. Both polar (ethylene glycol-adipate-polyester) (19) and nonpolar (Apiezon-L vacuum grease) stationary phases were used in the gas-liquid chromatographic columns with theoretical plate efficiencies of 2700 to 3600 measured with methyl stearate. Most of the analyses were done on polar stationary phase columns at 197°C. The identification of the primary and tertiary allylic alcohols

(prenols) from C_5 to C_{20}, and of the carboxylic acids (prenoic, or terpenoid acids) corresponding to the primary prenols, by the use of these two types of columns in gas-liquid chromatography has been described in detail (20). With the gas-liquid radiochromatographic technique one obtains a simultaneous recording of the analysis of a volatile mixture with a gas-density meter (21) together with that of the total C^{14}-content of any one component. The radiochromatographic record appears as discrete steps (integral shape record, Fig. 1) corresponding in location to the peaks recorded with the gas-density balance; the increment in counting rate in a given step indicates the total radioactivity associated with that particular fraction. The resolving power of the columns was such that not only could all the alcohols be completely separated but also their geometric isomers (e.g., *cis-trans-* and *trans-trans-*farnesol).

Phosphatase Preparations. Yeast inorganic pyrophosphatase was generously provided by Dr. P. Hele of this Unit; it had been purified by the method of Kunitz (22) to the stage immediately before crystallization, and stored at —20°C after precipitation with ammonium sulfate. For use the precipitate was dissolved in, and was dialyzed against, 0.02 M 2-amino-2-hydroxymethylpropane-1,3-diol-HCl (tris-HCl) buffer, pH 7.4. Incubations with this enzyme were made in 0.1 M tris-HCl buffer, pH 7.2 at 30°C for 15 minutes.

Purified calf-intestinal alkaline phosphatase was the product of C. F. Boehringer & Soehne, Mannheim, Germany. One hundred mg. of the freeze-dried preparation was dissolved in 5 ml. of 1 per cent bovine serum albumin solution and up to 0.5 ml. of this was used in incubations in 0.1 M tris-HCl buffer, pH 8.6.

Prostatic acid phosphatase was a 5-year-old preparation purified by the method of London and Hudson (23) from normal human prostates obtained from post-mortems. When prepared, the enzyme had a specific activity some three hundredfold greater than the best preparation of London and Hudson (23) and was free of proteolytic activity. The enzyme, precipitated with ammonium sulfate, had been stored at —20°C; it largely retained its activity. It was dissolved in and dialyzed before use against the same buffer as the yeast phosphatase. It was used in 0.1 M tris-acetate buffer, pH 5.7.

Snake venom *(Naja flava)* was obtained from the South African Institute for Medical Research, Johannesburg, and was used, after dissolving in 0.01 M $KHCO_3$, at pH 8.3 in 0.1 M tris-HCl buffer. It hydrolyzed the allyl pyrophosphates very slowly; at a concentration of 5 mg. per ml. only 10 per cent (0.15 μmole) of 1.5 μmoles of farnesyl pyrophosphate being hydrolyzed in 1.5 hours at 30°C. Even after 48 hours of action, only 85 per cent hydrolysis could be achieved with this preparation.

In all incubations with phosphatases Mg^{++} ions were present in a concentration of 5 mM. Hydrolysis of the allyl pyrophosphates with the prostatic and intestinal phosphatases was made in incubations at 37°C for 2 to 3 hours which were then continued at room temperature (22°C) for 16 to 20 hours to ensure complete hydrolysis. Free alcohols released during these incubations were extracted with petroleum ether after adjusting the pH of the incubations to about 10 and after the addition of 2 mg. of carrier alcohols.

Other Methods. Protein concentrations were determined with the biuret method (24). Inorganic orthophosphate was measured by the method described by London and Hudson (23); inorganic pyrophosphate is not detected by this method.

Materials. The sources of most of the purified coenzymes (TPN, DPN, ATP, and G-6-P) were given in a previous communication (25). Purified TPNH (enzymically reduced) and G-6-P dehydrogenase were the products of C. F. Boehringer and Soehne. Bovine plasma albumin, GSH, EDTA, BAL, and N-ethylmaleimide were obtained from L. Light and Co., Colnbrook, England.

The various inorganic salts and organic solvents and reagents were all analytical grade chemicals. Collidine (2,3-dimethyl pyridine) and petroleum ether were redistilled before use.

TABLE 1. SYNTHESIS OF ALLYL PYROPHOSPHATES FROM DL-MEVALONATE-2-C^{14} WITH SOLUBLE ENZYME FRACTION OF RAT LIVER*

Enzyme System	Allyl Pyrophosphates
	cpm.
Complete	30,220
No Mg^{++}	19
No ATP; + ADP (30 μmoles)	2,400
+ TPNH† (2 μmoles)	27,690
+ TPN (2 μmoles)	25,130
+ TPNH† + DPNH (2 μmoles each)	25,170

* Anaerobic incubation for 1 hr. at 37°C. The complete system contained: F_{30}^{60}-enzymes, 25 mg. protein/ml.; 7.5 mM ATP; 5.0 mM $MgCl_2$; 0.1 M potassium hydrogen phosphate buffer, pH 7.4; 0.01 M NaF; and 5 μmoles of DL-mevalonate-2-C^{14} (140,000 cpm. at negligible thickness), in a total volume of 4 ml.

† Generated with G-6-P and glucose-6-phosphate dehydrogenase present in the F_{30}^{60}-preparation.

DL-Mevalonic acid-2-C^{14} lactone was purchased from the Radiochemical Centre, Amersham, Bucks, England, and had been prepared by the method of Cornforth *et al.* (13). It was diluted with recrystallized, unlabeled mevalonic acid lactone before use, to give a solution with a specific activity of 0.2 mc. per mmole. As the lactone is inactive in enzyme reactions (10), the potassium salt of mevalonic acid was prepared by mild hydrolysis with KOH. Levorotatory 5-phosphomevalonate-2-C^{14} (26) was prepared by Levy and Popják (10) with purified liver mevalonic kinase; its specific activity was 32,000 cpm. per μmole (0.2 μc. per μmole) counted at negligible thickness.

Specimens of dimethylallyl alcohol, nerol (*cis*-geraniol), farnesol, nerolidol, and of squalene were provided by colleagues acknowledged at the end of this paper. Linalool was the product of A. Boake Roberts and Co., London, England, whose product "Meranol," which is pure geraniol, was also used. The sources and preparation of the various prenoic acids are described elsewhere (20).

RESULTS

Enzymic Synthesis of Allyl Pyrophosphates. When the F_{30}^{60}-enzymes were incubated with DL-mevalonate-2-C^{14}, ATP, and Mg^{++} ions aerobically or anaerobically, in the presence or absence of reduced pyridine nucleotides, only traces of radioactivity could be extracted with petroleum ether from the saponified incubation mixture. After acidification of the alkaline digests followed by neutralization, however, C^{14}-labeled neutral substances could be readily extracted. The results of a typical experiment, shown in Table 1, demonstrate that only ATP and Mg^{++} ions, and no pyridine nucleotides, are needed for the formation of these substances. With DL-mevalonate as substrate, the yield has varied from 20 to 40 per cent. With the optically active (-)5-phosphomevalonate as substrate, however, the yield was 75 to 80 per cent.

Gas-liquid radiochromatographic analyses of the neutral products extractable with petroleum ether, after acidification of the incubation mixtures, revealed a number of volatile substances different from any hitherto recognized animal product. Their origin from mevalonate suggested that they might be isoprenoid substances containing the characteristic grouping: $-CH_2-C(CH_3)=CH-CH_2$. Subsequently they were identified as C_5, C_{10}, and C_{15} allylic alcohols, since the radioactive fractions had, on columns with polar and nonpolar stationary phases, retention volumes indistinguishable from those of authentic marker substances added to the biosynthetic specimens (see further under Identification of the Allyl Alcohols).

It followed from the mode of isolation of the alcohols that they did not accumulate in the enzyme incubations in the free form, but as derivatives resistant to alkaline hydrolysis and very unstable in the presence of acid.

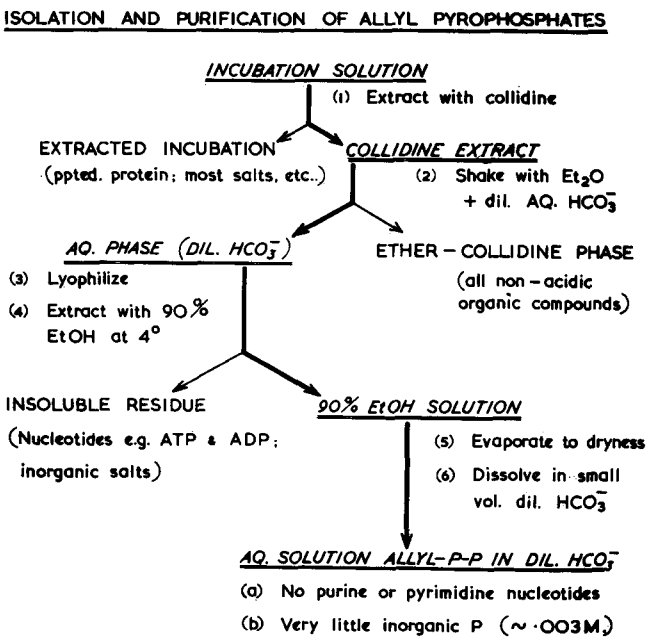
Further experiments showed that iodoacetamide in concentrations of 5 and 10 mM prevented completely the formation of these acid-labile derivatives of the allylic alcohols in incubations identical with the complete system shown in Table 1.

In all experiments on the synthesis of the acid-labile derivatives of the allylic alcohols with the F_{30}^{60}-enzymes, the incubations were made anaerobically and contained the same components, in the same concentrations, as listed in Table 1 for the complete enzyme system. When large-scale experiments were made for preparative purposes, multiples of this basic 4 ml. incubation mixture were set up and the incubations lasted for 2 hours, with additional amounts of ATP (5 μmoles per ml.) added at the end of the first hour.

Isolation and Partial Purification of Derivatives of Allylic Alcohols. The derivatives of the allylic alcohols could be extracted in an unhydrolyzed form from the enzymic incubations with collidine. A 28 ml. reaction mixture was set up with F_{30}^{30}-enzymes with 35 μmoles of DL-mevalonate-2-C^{14} as substrate. After 2 hours of incubation, a 4 ml. sample was assayed in the usual way (after saponification, acidification, etc.) for the amount of allylic derivatives formed; 40,980 cpm., equivalent to a conversion of 1.46 μmoles of mevalonate, were found in allylic alcohols in this 4 ml. sample. The remaining 24 ml. of the incubation was extracted three times with 20 ml. of collidine, the aqueous phase and the precipitated protein being separated by centrifugation. To the combined collidine extracts (75 ml.) 150 ml. of 0.02 N aqueous KOH and 100 ml. of ether were added in a separatory funnel and the mixture shaken. After separation of the phases, the ether-collidine layer was drawn off, and the extraction of collidine remaining in the aqueous phase completed by three further extractions with ether (100 ml. each). The combined ether extracts contained no radioactivity. The yellowish aqueous phase (86 ml.) contained all the radioactivity of the initial collidine extract. After removing the ether dissolved in the aqueous phase with a stream of nitrogen, the preparation was dried from the frozen state to yield a fine brownish powder. This powder was extracted in small portions with a total of 10 ml. of 80 per cent ethanol. This ethanolic solution, clarified by centrifuging, was

concentrated to 2 ml. under a stream of N_2 at room temperature (allyl pyrophosphate preparation No. 1). Extraction of this concentrate with petroleum ether removed only small amounts of radioactivity (equivalent to the C^{14}-content of about 0.001 μmole of mevalonate). One-fifth of the aqueous solution (after extraction with petroleum ether) was acidified with H_2SO_4 (pH about 1) and then after about 30 minutes was made alkaline and extracted again with petroleum ether. This extract now contained 48,827 cpm. Thus, through the whole process of collidine extraction, etc., $5 \times 48{,}827 = 2.441 \times 10^5$ cpm. of acid labile C^{14}-compounds were obtained from 24 ml. of incubation, corresponding to the conversion of 8.7 μmoles of mevalonate. From the direct analysis of the 4 ml. aliquot of the original 28 ml. incubation, the expected value was 2.459×10^5 cpm. Gas-liquid radiochromatographic analysis of the petroleum ether extracts of the acidified 4 ml. aliquot of the original incubation and of the final petroleum ether extract of the specimen prepared through collidine extraction revealed the same allylic alcohol components in identical ratios.

The method of isolation and partial purification of the derivatives of the allylic alcohols given in Scheme 1 is based on experience gained during the experiment



SCHEME 1. Flow sheet of the purification of allyl pyrophosphates.

just described, and was used during later experiments. The progress of the purification is indicated by the italics and heavy arrows. The compounds were first extracted from the enzymic incubations with collidine, and their aqueous solution prepared by shaking the collidine extract with diethyl ether and dilute aqueous bicarbonate (in preference to KOH). The solubility of the compounds in water at neutral or slightly alkaline pH is considerably greater than in collidine, so that several extractions with excess volume of collidine were necessary to achieve a quantitative extraction of the products from the incubation mixtures. They are practically insoluble in an ether-collidine mixture (2:1, v/v) so that on the addition of 2 volumes of ether and 0.5 volumes of 0.01 M aqueous $KHCO_3$ to the collidine extract, the compounds could be quantitatively transferred into the aqueous phase. Contaminating nucleotides and salts were then removed by precipitation of these at 4°C by the addition of ethanol to a final concentration of 90 per cent. After evaporation of the ethanol at room temperature under reduced pressure, the compounds were dissolved in 0.01 M aqueous $KHCO_3$ and stored at —20°C.

For further studies a large-scale, 2-hour incubation (75×4 ml.) was set up with 8 g. of F^{60}_{30}-enzymes and with a total of 300 μmoles of (-)5-phosphomevalonate-2-C^{14} as substrate. The derivatives of the allylic alcohols were isolated from this incubation according to the procedure outlined in Scheme 1; assay of an aliquot of the incubation mixture showed that 240 μmoles of the substrate added were converted into these substances. Unless stated otherwise, the experiments still to be described were made with this preparation (allyl pyrophosphate preparation No. 3).

Identification of the Acid-Labile Derivatives of Allylic Alcohols as Pyrophosphate Esters. Lynen *et al.* (4) noted that farnesyl pyrophosphate when exposed to 5 per cent cold trichloroacetic acid decomposed into inorganic pyrophosphate and an organic residue. The instability in the presence of acid of the derivatives of the allylic alcohols formed in liver enzyme incubations suggested that these also might be pyrophosphate esters. To test this possibility an aqueous solution of the compounds (preparation No. 3) was first acidified in order to liberate the free alcohols (see below). The solution was then neutralized, and the alcohols extracted with petroleum ether. The number of micromoles of alcohols released was calculated from the C^{14}-content of the petroleum ether extract, the gas-liquid radiochromatographic analysis of the alcohols, and from the known specific activity of the 5-phosphomevalonate-2-C^{14} from which they had been synthesized. The specific activity (counts per minute per μmole) of the C_{15}-alcohols (farnesol and nerolidol) was taken as three times, and

that of the C_{10}-alcohols as twice that of the 5-phosphomevalonate. One-half of the aqueous phase extracted with petroleum ether was analyzed directly for inorganic orthophosphate. The other half was incubated with yeast inorganic pyrophosphatase—which splits inorganic pyrophosphate into orthophosphate—and then also analyzed for orthophosphate. As is shown in Table 2, incubation with pyrophosphatase

TABLE 2. IDENTIFICATION OF ALLYL ALCOHOL DERIVATIVES AS PYROPHOSPHORIC ACID ESTERS*

Treatment	μmole Orthophosphate /μmole Alcohol
(*a*) Acidify and then neutralize	1.4
(*b*) Acidify and neutralize + pyrophosphatase treatment	3.0
(*c*) Pyrophosphatase alone†	1.8

Pyrophosphate released by acid = $0.5 \times (b - a)$ = 0.8 μmole/μmole alcohol

* Each analysis was made on 0.2 μmole of allyl alcohol derivatives (prep. No. 3). See also text.

† It is probable that the small increase in orthophosphate as compared to (*a*) is due to slight hydrolysis of allyl pyrophosphates by yeast phosphatase. These compounds have been hydrolyzed, although sometimes quite slowly, by every phosphatase tested so far.

released an additional 1.6 μmoles of orthophosphate (equivalent to 0.8 μmole of pyrophosphate) per μmole of alcohol released. The amount of orthophosphate listed in line *a*, 1.4 μmoles per μmole of alcohol, is considered to represent the inorganic phosphate content of the partially purified specimen. To prove that the effect of pyrophosphatase treatment was not due to the presence of preformed inorganic pyrophosphate in the solution, a third aliquot was incubated with the pyrophosphatase without previous acidification. As shown in line *c*, this resulted in only a very small increase in the content of orthophosphate. These data therefore show that acidification releases almost 1 mole of inorganic pyrophosphate per mole of alcohol, from which we conclude that the original compounds are alcohol pyrophosphates.

Identification of the Allyl Alcohols. The organic moieties of the pyrophosphate esters were identified as allylic alcohols by gas-liquid radiochromatographic analysis. Samples of the pyrophosphate esters (allyl pyrophosphate preparations No. 1 and No. 3) were hydrolyzed with various phosphatases for sufficient length of time to ensure complete hydrolysis (see Methods). The enzymic hydrolysis was judged complete when the amount of C^{14} extractable with petroleum ether after such hydrolysis was identical with the amount of C^{14} extractable after acid hydrolysis. Figure 1 illustrates typical gas-liquid radio-

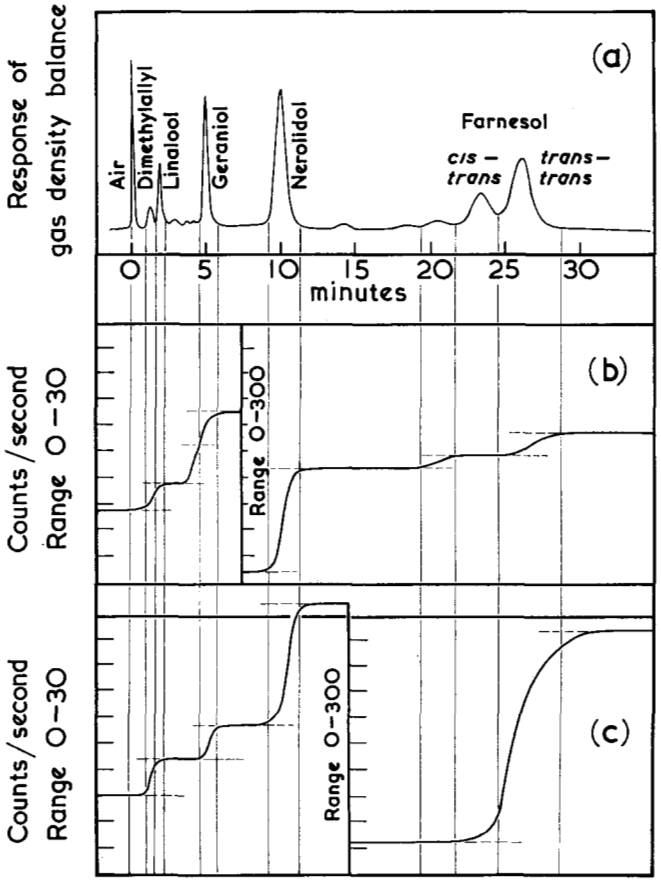


FIG. 1. Gas-liquid radiochromatographic analysis of the alcohols released from an aqueous solution of allyl pyrophosphates (see text). Ethylene glycol-adipate polyester stationary-phase column; 197°C. Record (*a*) shows the analysis of a reference mixture of unlabeled alcohols added as carrier; record (*b*) shows the simultaneous radiochromatographic analysis of the alcohols released by acid hydrolysis; and record (*c*) shows the alcohols released by enzymic hydrolysis, with prostatic acid phosphatase.

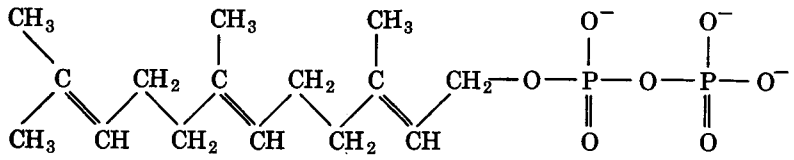
chromatographic analyses of allylic alcohols on ethylene glycol-adipate polyester column: (*a*) is the analytical record (gas-density meter response) of authentic marker substances added to the biosynthetic specimens; (*b*) and (*c*) are the radiochromatographic records of the organic components of allyl pyrophosphate preparation No. 3 hydrolyzed by acid and by prostatic acid phosphatase respectively. Records (*b*) and (*c*) were obtained of course in two separate runs (with identical markers), but for purposes of illustra-

tion were combined into one figure. In records (*b*) and (*c*) certain radioactive fractions correspond exactly with the analytical peaks shown in record (*a*). Since similar correspondence was found during analyses on another type of column (stationary phase Apiezon-L vacuum grease), the radioactive components may be assigned with confidence to some of the marker substances.

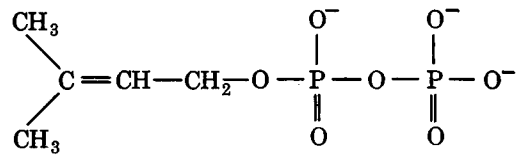
After hydrolysis with prostatic phosphatase, almost 90 per cent of the radioactivity was associated with *trans-trans* farnesol, about 6 per cent with the tertiary C_{15}-alcohol nerolidol, and smaller amounts with geraniol and 3,3-dimethylallyl alcohol (Fig. 1*c*). There were also present small radioactive fractions with retention volumes greater than that of farnesol. After hydrolysis with acid, however (Fig. 1*b*), a distinctly different pattern was obtained. Here, as after enzymic hydrolysis, about 95 per cent of the radioactivity was associated with the C_{15}-alcohols farnesol and nerolidol, but there was a striking difference in that the amount of nerolidol was now almost seven times greater than that of farnesol. Acid hydrolysis has also consistently resulted in the appearance of dimethylallyl alcohol, linalool, nerol (*cis*-geraniol), geraniol, a material with a retention volume on polyadipate columns of 1.85 times that of farnesol (corresponding to the C_{20}-tertiary alcohol geranyllinalool), and occasionally also a substance with a retention volume of 3.56 times that of farnesol.

Further analyses of the alcohol portions of the allyl pyrophosphates have been made after hydrolysis of these compounds with other phosphatases. In each instance the aqueous solution of the allyl pyrophosphates was extracted with petroleum ether before hydrolysis with the phosphatase. Samples of allyl pyrophosphate preparation No. 3 were also hydrolyzed

FORMULAE



(I)



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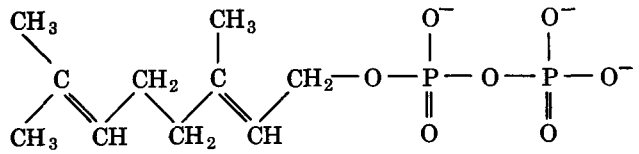

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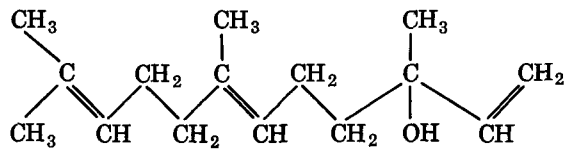

(IV)

with intestinal alkaline phosphatase and with the phosphatase present in liver microsomes (the latter in 0.1 M tris-HCl buffer pH 7.4; see below). With each of these two phosphatase preparations, the same alcohols were found as after hydrolysis with prostatic acid phosphatase. The farnesol-to-nerolidol ratio differed somewhat from one preparation to another, for the activity of nerolidol was only 1 to 2 per cent of the total after intestinal alkaline phosphatase hydrolysis, whereas after hydrolysis with microsomes, it was the same as after prostatic acid phosphatase.

A sample of allyl pyrophosphate preparation No. 1 was hydrolyzed at pH 8.3 with snake venom phosphatases for 24 hours, in which time only 75 per cent hydrolysis was achieved. Analysis of the alcohols so released again revealed the presence of dimethylallyl alcohol, geraniol, nerolidol, and farnesol. Farnesol plus nerolidol represented 80 per cent of the total radioactivity, and the amount of farnesol was six times that of nerolidol.

One further technique, suggested by Dr. J. W. Cornforth, was employed to identify the alcohols of the pyrophosphate esters and in particular to decide whether nerolidol was a genuine enzymic product or not. A portion of allyl pyrophosphate preparation No. 1, containing 7,000 cpm. of C^{14}, was freeze-dried and then refluxed for 75 minutes with 2 ml. of 0.78 M ethereal solution of $LiAlH_4$; a method used by Karrer and Jucker (27) for the fission of cetylphosphate. After decomposition of the $LiAlH_4$ by careful addition of water, the layer of ether was drawn off, and the aqueous phase extracted three times with small portions of ether. The combined ether extracts were washed three times with 2 ml. of water and then the ether dried with $MgSO_4$. A total of 4,000 cpm. of C^{14} were found in this ether extract. Gas-liquid radiochromatographic analysis revealed the presence of two hitherto unidentified components (? hydrocarbons) and of farnesol and nerolidol, the ratio of the amounts of the latter two being 5:1.

These data therefore indicate that under the conditions employed for the biosynthesis of allyl pyrophosphates, the major product obtained was farnesyl pyrophosphate (formula I). In preparation No. 3 this compound represented more than 85 per cent of the total C^{14}-activity of the reaction products. 3,3-Dimethylallyl pyrophosphate (formula II) and geranyl pyrophosphate (formula III) are also always present, but in much smaller amounts. A small amount of nerolidol (formula IV) was always found in the analyses of the alcohols released by methods other than acidification, but the farnesol-to-nerolidol ratio varied slightly from one method to another. The question of the existence of nerolidyl pyrophosphate as an enzymic product is considered under Discussion. The differences between the analyses of the alcohols released by acid hydrolysis and those released by enzymic hydrolysis are undoubtedly due to rearrangements occurring during acid hydrolysis, and will also be discussed later.

Stability of the Allyl Pyrophosphates. For a study of the stability of the allyl pyrophosphates at various pH's, identical samples (containing 1,000 cpm.) of allyl pyrophosphate preparation No. 3 were added to 0.1 M buffers (3 ml.) of various pH's. After standing at 22°C for 6 hours, the mixtures were made alkaline (pH 10) with aqueous KOH and were extracted (after addition of 2 mg. of a mixture of allylic alcohols) with petroleum ether. The C^{14}-counts found in the petroleum ether extract were expressed as a percentage of the total C^{14}-counts that were released by strong acid and were taken to represent the degree of cleavage of the allyl pyrophosphates. The pH ranges 2.0 to 4.0 were covered by potassium phthalate-HCl buffers, pH 4 to 6 by sodium acetate buffers, and pH 6 to 8 by potassium hydrogen phosphate buffers. Since over 85 per cent of the allyl pyrophosphate preparation No. 3 consisted of farnesyl pyrophosphate (Fig. 1*c*), the data shown in Figure 2 are thought to depict the properties of farnesyl pyrophosphate. Above pH 5.5

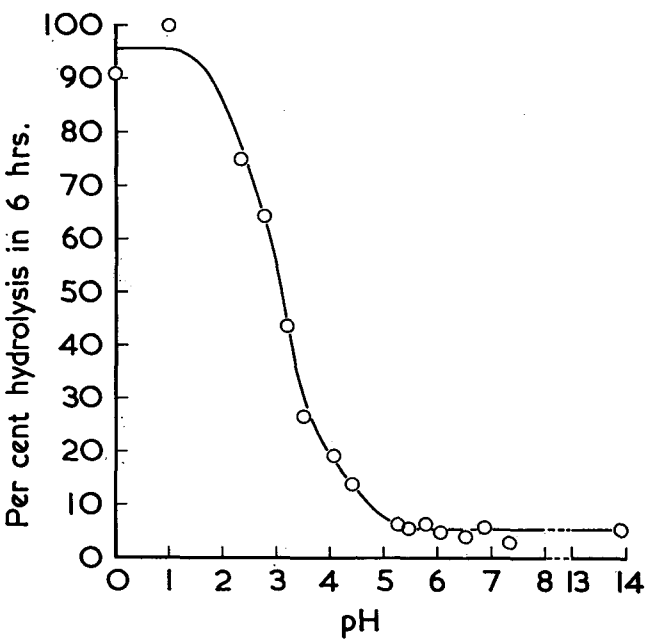

FIG. 2. Instability of farnesyl pyrophosphate as a function of pH. The percentage hydrolysis observed in 6 hours is plotted against the pH (see text for details of experimental procedure).

the compound is relatively stable, although it does hydrolyze very slowly even in alkaline solution. Below pH 5, however, farnesyl pyrophosphate hydrolyzes with a rate which varies inversely with pH. The implications of the sigmoid shape of the curve will be considered later. At any pH, hydrolysis at room temperature continues until it has gone to completion. Figure 3 is a plot of per cent hydrolysis as a function

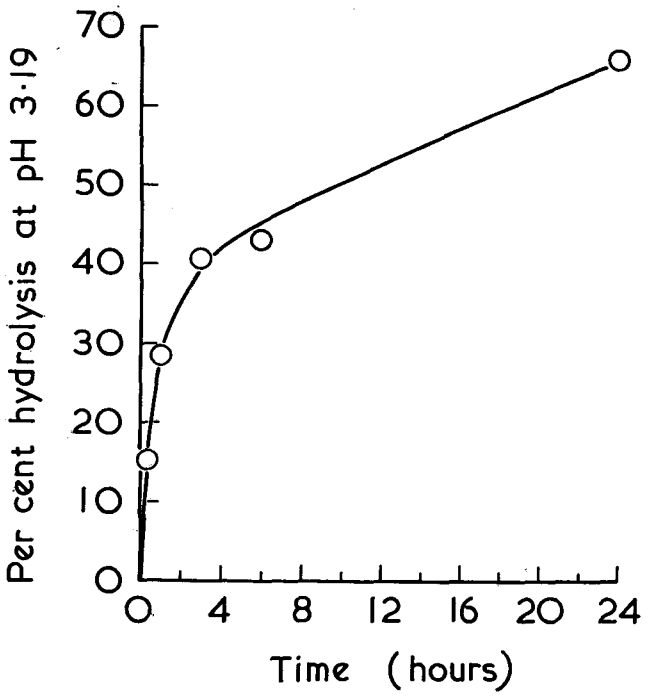

FIG. 3. Rate of hydrolysis of farnesyl pyrophosphate in 0.1 M phthalate buffer, pH 3.19, at 22°C.

of time at pH 3.19, a point near the center of the sigmoid curve in Figure 2. At pH 4, about 5 days are needed for complete hydrolysis.

Farnesyl pyrophosphate is fairly stable when heated in alkaline or neutral solution. Solutions of this compound in 0.1 N KOH were heated for 1 hour at 100°C, 80°C, and 60°C. This resulted in a hydrolysis of 15 per cent, 10 per cent, and 8 per cent, respectively. Heating a solution in 0.1 M potassium hydrogen phosphate buffer, pH 7.4 (measured at 22°C), at 100°C for 1 hour caused 28 per cent hydrolysis. When kept frozen at —20°C, however, farnesyl pyrophosphate was very stable (less than 2 per cent hydrolysis in 3 months).

Synthesis of Squalene from Farnesyl Pyrophosphate. The conversion of farnesyl pyrophosphate into squalene was studied in several experiments. Allyl pyrophosphate preparation No. 3 was used as substrate in all these experiments. The optimum reaction mixture for the anaerobic conversion of farnesyl pyrophosphate into squalene contained in a final volume of 1 ml. washed microsomes, 0.05 ml.; $MgCl_2$, 5 mM; TPNH or DPNH, 1 to 3 mM; nicotinamide 30 mM; NaF, 10 mM; potassium phosphate buffer, pH 7.4, 0.1 M; plasma albumin, 2 mg.; and C^{14}-farnesyl pyrophosphate, 0.03 to 0.1 μmole. This mixture will be referred to as the "complete squalene synthesizing system." Unless otherwise specified, TPNH was always generated in the incubation mixture by the addition of TPN, G-6-P, and purified glucose-6-phosphate dehydrogenase. Nicotinamide was added in order to inhibit microsomal destruction of the pyridine nucleotides and sodium fluoride to inhibit a potent microsomal phosphatase which hydrolyzes the allyl pyrophosphates. Table 4 demonstrates that this phosphatase

TABLE 3. COMPONENTS OF THE SQUALENE SYNTHESIZING SYSTEM OF RAT LIVER*

Washed microsomes	0.05 ml.
$MgCl_2$	5 mM
TPNH† or DPNH	1-3 mM
Nicotinamide	30 mM
NaF	10 mM
Potassium PO_4 buffer, pH 7.4	0.1 M
Plasma albumin	2 mg.
C^{14}-farnesyl pyrophosphate	0.03–0.1 μmole

* Complete anaerobic incubation mixture contained in 1.0 ml.
† Usually generated with G-6-P + glucose-6-phosphate dehydrogenase.

TABLE 4. HYDROLYSIS OF ALLYL PYROPHOSPHATES BY RAT LIVER MICROSOMES*

Buffer	Additions	Hydrolysis
		per cent
Tris-HCl	None	7
Tris-HCl	Microsomes, 0.05 ml.	43
Phosphate	Microsomes, 0.05 ml.	16
Tris-HCl	Microsomes, 0.05 ml.; NaF 10 μmoles	11
Phosphate	Microsomes, 0.05 ml.; NaF 10 μmoles	8

* Each incubation contained in a final volume of 1 ml. 3700 cpm. of C^{14}-labeled allyl pyrophosphates (0.03 μmole of farnesy pyrophosphate) 2 mg. bovine plasma albumin, 5 mM $MgCl_2$, 30 mM nicotinamide, and 0.1 M buffer pH 7.4. Incubations under N_2 at 37°C for 1 hr.

was strongly inhibited by the combination of phosphate buffer and fluoride. With the complete squalene synthesizing system containing TPNH, the yield of squalene from farnesyl pyrophosphate varied from 50 to 80 per cent. The squalene formed was identified by chromatography on alumina, followed by formation of the thiourea adduct.

The pyridine nucleotide requirements for this system are shown in Table 5. When these nucleotides were not added, or TPN alone was used, squalene was not formed. With TPNH, however, there was an excellent yield of squalene; with DPNH the yield was also good, but less than with TPNH.

TABLE 5. NEED FOR REDUCED PYRIDINE NUCLEOTIDES IN THE SYNTHESIS OF SQUALENE FROM FARNESYL PYROPHOSPHATE*

Addition	Squalene
	cpm.
None	70
TPN (1 μmole)	120
TPNH (1 μmole†)	2670
DPNH (1 μmole)	1900

* Each tube contained in 1 ml. the complete squalene synthesizing system (Table 3) without TPNH (or DPNH), and with 3300 cpm. (0.03 μmole) of C^{14}-farnesyl pyrophosphate. Incubations under N_2 for 1 hr. at 37°C.

† Generated from 1 μmole of TPN with 3 μmoles of G-6-P and purified glucose-6-phosphate dehydrogenase.

The need for the presence of a divalent cation was demonstrated by using microsomes which had been dialyzed for 2 hours against a large volume of 0.02 M phosphate buffer containing 1 mM EDTA. Purified TPNH was used in these experiments. The data given in Table 6 demonstrate that in the absence of a divalent cation, the yield of squalene was very small (less than 10 per cent). On the addition of either Mg^{++}, Mn^{++}, or Co^{++}, however, a good yield of squalene (55 to 60 per cent) was obtained. Nickel ions were able to satisfy partially the cation requirement, but Cu^{++}, and Ca^{++} ions were inhibitory. That this system requires a divalent cation could also be inferred from the fact that in the presence of an excess EDTA the synthesis of squalene was completely inhibited (Table 8).

The rate of squalene formation in this system is shown in Figure 4. These studies also provide the best comparison between the effectiveness of TPNH and DPNH, since identical concentrations of purified reduced pyridine nucleotide (2 μmoles per ml.) were used. It is apparent that the reaction shows very little specificity for either TPNH or DPNH.

TABLE 6. DIVALENT CATION REQUIREMENT FOR SQUALENE SYNTHESIS FROM FARNESYL PYROPHOSPHATE*

Addition	Squalene
	cpm.
None	372
Mg^{++} 5 μmoles	2320
Mn^{++} 5 μmoles	2220
Co^{++} 5 μmoles	2340
Ni^{++} 5 μmoles	735
Ca^{++} 5 μmoles	120
Zn^{++} 5 μmoles	87
Cu^{++} 5 μmoles	22

* Each tube contained in 1 ml. the complete squalene synthesizing system (Table 3) without $MgCl_2$ with 4000 cpm. (0.036 μmole) of C^{14}-farnesyl pyrophosphate, 1 μmole of TPNH, and 0.1 ml. of microsomes dialyzed against 1 mM EDTA. Incubations under N_2 for 1 hr. at 37°C.

Evidence has been obtained that only the farnesyl pyrophosphate present in the substrate solution of allyl pyrophosphates was used for squalene synthesis in these experiments.[1] The fact that up to 80 per cent yield of squalene could be obtained from a solution, more than 85 per cent of which was farnesyl pyrophos-

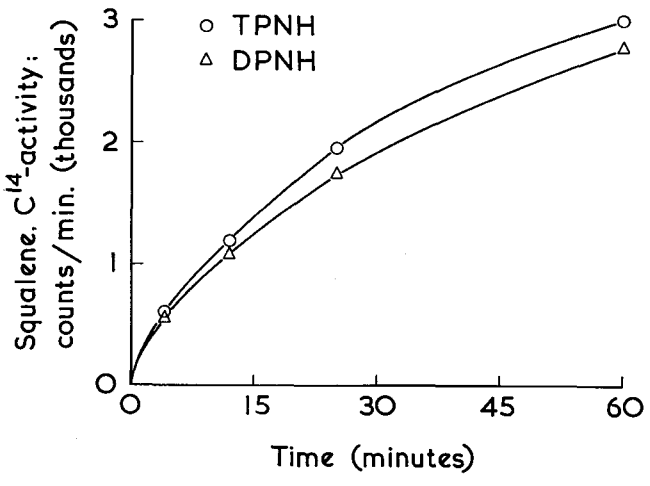

FIG. 4. Rate of squalene synthesis from farnesyl pyrophosphate. Aliquots (0.5 ml.) withdrawn at time intervals shown from 3 ml. complete anaerobic incubation mixtures (Table 3) containing 6000 cpm. of farnesyl pyrophosphate/ml., and 2 μmoles/ml. of either TPNH or DPNH.

[1] Nerolidyl pyrophosphate, if it were a true enzymic product in this solution (see Discussion), would probably have also participated in this reaction.

phate, indicates, of course, that most of the squalene must be synthesized from this compound. These data do not, however, establish that the small amounts of dimethylallyl pyrophosphate and geranyl pyrophosphate do not also participate in this reaction under the conditions employed. In order to investigate this question, the saponified incubation mixtures which had contained TPNH and DPNH in the experiment recorded in Table 5 (i.e., the incubations that gave a good yield of squalene) after extraction of the squalene were acidified, and the liberated alcohols extracted after neutralization. These alcohols, which represented the unused allyl pyrophosphates in these incubations, were analyzed by gas-liquid radiochromatography. The ratio of the amount of C_5- plus C_{10}-alcohols to that of the C_{15}-alcohols present was found to be 0.163. The value of this ratio for the original substrate solution (Fig. 1) was 0.057. These data therefore demonstrated a considerable enrichment of the shorter chain alcohol pyrophosphates in the solutions in which squalene synthesis took place. It can be inferred therefore that only the C_{15}-compounds were used in this reaction.

The Effect of pH on Squalene Synthesis from Farnesyl Pyrophosphate. The effect of pH on the synthesis of squalene from farnesyl pyrophosphate was studied in the pH range 6.0 to 8.6. This range was covered by potassium phosphate (6.0 to 7.4) and tris-HCl (7.4 to 8.6) buffers. Separate experiments were made with TPNH and DPNH. The results showed (Fig. 5) that the optimum pH was 7.4, although squalene synthesis proceeded well over the entire range studied. The explanation for the differences noted with TPNH and DPNH is obscure.

Effect of Soluble Enzymes. When dialyzed soluble enzymes were added to the complete squalene synthesizing system, the yield of squalene was consistently reduced, and under these conditions there occurred some conversion of the allyl pyrophosphates into carboxylic acids. The results shown in Table 7 are representative of this effect, and also demonstrate the fact that in the presence of soluble enzymes the difference between TPNH and DPNH in promoting squalene synthesis is considerably enhanced, with TPNH acting as a much more effective coenzyme for squalene formation. The nature of the carboxylic acids formed in this system, the mechanism of their formation, and their physiologic role are at present under investigation and will be the subject of a future report.

Inhibitor Studies. A variety of compounds was tested to ascertain whether they might inhibit the synthesis of squalene in this system. Table 8 contains

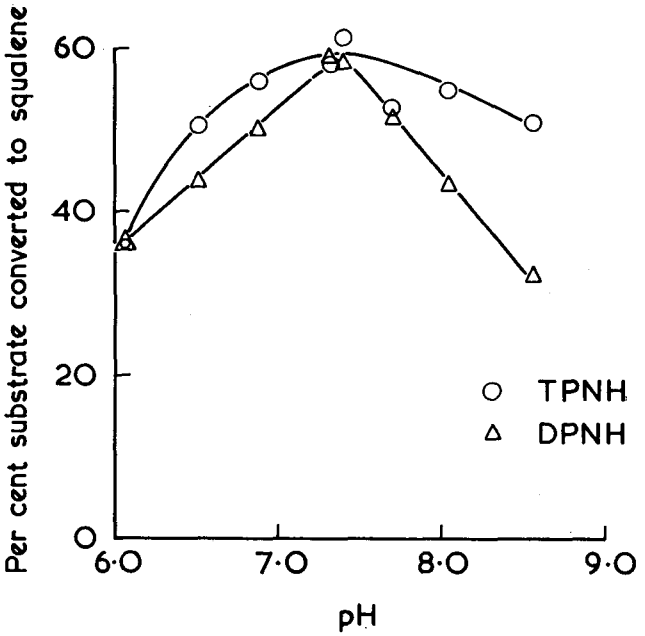

FIG. 5. Effect of pH on the synthesis of squalene from farnesyl pyrophosphate. One-hour complete anaerobic incubations at 37°C, in 0.1 M phosphate or tris buffer (see text).

the combined results of four sets of experiments. In each experiment a control, consisting of the complete squalene synthesizing mixture without inhibitor, was included. The per cent inhibition was calculated from the yield of squalene in the presence of the inhibitor, as compared to yield in the control for that experiment. The per cent inhibition is listed as "none" if the yield

TABLE 7. EFFECT OF SOLUBLE ENZYMES ON SQUALENE SYNTHESIS FROM FARNESYL PYROPHOSPHATE*

Additions	Squalene	Residual Allyl-PP	Carboxylic Acids
	cpm.	*cpm.*	*cpm.*
TPNH (1 μmole)†	6160	1400	160
DPNH (1 μmole)	4830	1570	120
F_{30}^{60}-enzymes (23 mg. protein) + TPNH†	4120	750	1500
F_{30}^{60}-enzymes (23 mg. protein + DPNH (1 μmole)	2230	720	2960

* Each tube contained 7500 cpm. of C^{14}-allyl pyrophosphates (7000 cpm., i.e., 0.064 μmole, of farnesyl pyrophosphate) and the complete squalene synthesizing system (Table 3) without TPNH or DPNH; the final volume was 1 ml. Incubations under N_2 for 1 hr. at 37°C.

† Generated from 1 μmole of TPN and 2 μmoles of G-6-P with glucose-6-phosphate dehydrogenase.

TABLE 8. INHIBITORS OF SQUALENE SYNTHESIS FROM FARNESYL PYROPHOSPHATE*

Additions†	Inhibition
μmoles	*per cent*
GSH (5)	none
BAL (10)	none
GSSG (10)	13
PCMB (0.01)	none
PCMB (0.1)	63
PCMB (1)	98
PCMB (2.5) + ascorbate (10)	100
PCMB (2.5) + GSH (5)	13
N-ethyl maleimide (1)	54
N-ethyl maleimide (3)	75
Iodoacetamide (0.2)	none
Iodoacetamide (2)	none
Iodoacetamide (10)	none
Na arsenite (0.5)	16
Na arsenite (3)	25
Na arsenite (3) + GSH (10)	none
Na arsenite (3) + BAL (10)	none
EDTA (20)	100
$Cu(NO_3)_2$ (1)	98
$CaCl_2$ (1)	61
$NiSO_4$ (1)	47
Zn acetate (1)	26
$FeSO_4$ (1)	15
$CoCl_2$ (1)	13
$MnCl_2$ (1)	none
KCN (1)	none

* Each tube contained in 1 ml. the complete squalene synthesizing system with 1 μmole of TPNH and 0.03 μmole of C^{14}-farnesyl pyrophosphate. In these experiments 1 μmole of TPN was first reduced with 3 μmoles of G-6-P and glucose-6-phosphate dehydrogenase; the components of the squalene synthesizing system and the inhibitors were added afterward (see also text). Incubations under N_2 for 1 hr. at 37°C.

† Numbers in parentheses indicate μmoles of inhibitors added.

of squalene in that tube was 90 per cent or more of the control yield. It is apparent from Table 8 that a variety of substances inhibit this system. PCMB is a very potent inhibitor, and its effects could be almost completely abolished by the addition of GSH. N-ethyl maleimide also inhibited the reaction, but the third SH-inhibitor, iodoacetamide, did not. Among the divalent cations tested (all in the presence of 5 mM Mg^{++}) Cu^{++} was the most potent inhibitor, squalene synthesis being completely abolished in its presence. Significant inhibition was also observed with Ca^{++}, Ni^{++}, and Zn^{++}. The two ions which were able to replace Mg^{++} in satisfying the cation requirement for squalene synthesis (Table 6) either exerted no (Mn^{++}) or only borderline (Co^{++}) inhibitory effects.

Free Alcohols. Studies were performed (together with Dr. J. Christophe) to ascertain whether the free allylic alcohols might be either phosphorylated and/or converted into squalene by rat liver enzymes. Experiments were made with C^{14}-alcohols released from the pyrophosphates by hydrolysis with prostatic phosphatase and with those obtained after acidification. Incubation mixtures were set up containing microsomes with or without dialyzed, unfractionated soluble enzymes (F_0^{100}-enzymes), ATP, and TPNH, in various combinations, and together with $MgCl_2$, nicotinamide, NaF, and phosphate buffer in the usual concentrations. After a 1-hour anaerobic incubation at 37°C, the solutions were analyzed for squalene, free alcohols, and allyl pyrophosphates. In no instance was more than a trace of radioactivity (less than 2 per cent of the total activity in all cases) found in either the squalene or the allyl pyrophosphate fractions. These results indicate that if phosphorylation of the free alcohols and/or squalene synthesis from the free alcohols (mainly farnesol) occurs at all, it is insignificantly small.

DISCUSSION

The results of the experiments reported here indicate that the reactions of squalene biosynthesis in rat liver are similar to those occurring in yeast cells. It has been demonstrated that in the yeast cell these reactions include the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate, the condensation of these two compounds to give geranyl pyrophosphate and the condensation of geranyl pyrophosphate with isopentenyl pyrophosphate to yield farnesyl pyrophosphate (3, 6). The present findings, that a fraction of soluble enzymes from rat liver homogenates catalyzes the formation of farnesyl pyrophosphate from mevalonate or from 5-phosphomevalonate in excellent yield, and that dimethylallyl pyrophosphate and geranyl pyrophosphate are also always formed during this process, indicate that these same reactions occur in the liver cell. It has also been shown that iodoacetamide completely inhibited the synthesis of allyl pyrophosphates from 5-phosphomevalonate. Since iodoacetamide does not inhibit mevalonic (10) and phosphomevalonic kinase from liver,[2] it must inhibit either diphosphomevalonic decarboxylase or isopentenyl pyrophosphate isomerase. Lynen *et al.* (6) have shown that in the yeast system only the latter enzyme is inhibited by iodoacetamide.

[2] Hellig and Popják, unpublished observations.

It thus seems likely that it is this same enzyme which is inhibited in the liver system also.[3]

There can be no doubt that farnesyl pyrophosphate is the major (or only) sesquiterpenoid intermediate in squalene biosynthesis, since squalene can be obtained from it in yields as high as 80 per cent. In both the liver and the yeast cell the reactions involved in the formation of farnesyl pyrophosphate (and of the other allyl pyrophosphates) from mevalonate are catalyzed by enzymes found in the soluble fraction of the disrupted cells. The only cofactors needed for the synthesis of the allyl pyrophosphates from mevalonate or from 5-phosphomevalonate are ATP and a divalent cation. It was demonstrated in Bloch's laboratory (2, 3) that 3 moles of ATP are required for the formation of 1 mole of isopentenyl pyrophosphate from 1 mole of mevalonate, and both Chaykin *et al.* (2) and Lynen *et al.* (4) have shown that squalene synthesis can proceed from isopentenyl pyrophosphate without ATP. It follows, therefore, that in the soluble liver enzyme system the ATP is probably needed only for the formation of isopentenyl pyrophosphate and that the synthesis of geranyl and farnesyl pyrophosphates proceeds by the electrophilic condensation of dimethylallyl pyrophosphate and of geranyl pyrophosphate with isopentenyl pyrophosphate, as proposed by Lynen *et al.* (4), by Rilling and Bloch (28), and by Cornforth and Popják (29), and proved experimentally by Lynen and his colleagues (6).

The instability of the allyl pyrophosphates at acid pH as well as their reactivity in enzymic reactions is attributable to their chemical structure, as discussed by Cornforth (30). Allyl pyrophosphates, in common with other substances in which a potentially anionic group is in the α-position to a double bond, are electrophilic reagents and can be expected to lose the pyrophosphate ion rather readily. The electron deficient species $\left[\begin{array}{c} | \quad\quad | \quad\quad | \\ C \cdots CH \cdots C \\ | \quad\quad | \quad\quad | \end{array} \right]^+$ resulting from the elimination of the pyrophosphate ion, may be partially stabilized by resonance between two equivalent forms $\begin{array}{c} | \quad\quad | \\ C{=}CH{-}C^+ \\ | \quad\quad | \end{array}$ and $\begin{array}{c} | \quad\quad | \\ C^+{-}CH{=}C \\ | \quad\quad | \end{array}$. In the presence of water these two forms may yield, by the addition of a hydroxyl ion, either a primary or a tertiary alcohol. This is the mechanism of an allylic rearrangement, and is undoubtedly responsible for the conversion of a large part of farnesyl pyrophosphate to nerolidol during acid hydrolysis (detailed discussion in reference 31). The equilibria involved differ from one compound to another, for while most of the farnesyl pyrophosphate is converted to nerolidol, only a small fraction of the geranyl pyrophosphate is converted into the corresponding tertiary alcohol linalool. In addition, other rearrangements seem to occur during acid hydrolysis of the pyrophosphates, such as a small degree of *cis-trans* isomerization resulting in the appearance of some nerol from geranyl pyrophosphate. The allylic rearrangement of farnesyl pyrophosphate was also noted by Lynen *et al.* (6).

Evidence that it is the undissociated allylpyrophosphoric acid which is the unstable species is provided by the data shown in Figure 2. It will be noted that this plot of per cent hydrolysis in 6 hours against pH resembles a titration curve. Furthermore, the data shown in Figure 3 indicate that with longer periods of hydrolysis, this plot can be expected to move to the right, whereas with shorter time intervals, it will lie farther to the left (probably with associated minor changes in the shape of the curve). Now the value of pK_1 for the dissociation of a single hydrogen ion from farnesyl pyrophosphoric acid can be expected to be the same as that for pyrophosphoric acid, namely, 1.9. If it is indeed the undissociated acid which is the unstable species, it can be expected that with shorter and shorter periods of hydrolysis, the limiting position of the curve in Figure 2 will have pH 1.9 as its midpoint. At 6 hours the curve lies only one pH unit to the right. There is, of course, a good theoretical reason to expect the undissociated acid to be the unstable molecular species. The mechanism of elimination from an allyl pyrophosphate discussed above requires that the pyrophosphate moiety extract an electron from the organic portion of the molecule and leave as an anion. If the pyrophosphate portion of the molecule already bears a negative charge by virtue of the dissociation of one or more hydrogen ions, it will be much less likely to be able to extract still another negative charge (an electron) from the organic portion of the molecule.

Evidence for the correctness of these postulated mechanisms comes from the present demonstration of a quantitative relation between the amounts of inorganic pyrophosphate and of free alcohol liberated during acid hydrolysis of allyl pyrophosphates and from the observed allylic rearrangement of farnesyl pyrophosphate to nerolidol. Lynen *et al.* (4) demonstrated qualitatively (by paper chromatography) the appearance of inorganic pyrophosphate during acid hydrolysis of farnesyl-P^{32}-pyrophosphate biosynthesized from mevalonate and P^{32}-labeled ATP.

[3] Since this paper was submitted, it was found that in incubations of F_{30}^{50}-enzymes with mevalonate, ATP, Mg^{++}, and 5 mM iodoacetamide, isopentenyl pyrophosphate alone accumulated.

The data on the synthesis of squalene from farnesyl pyrophosphate demonstrate that the enzyme(s), which we propose to call the squalene synthetase system, is associated with the microsomes, and that the only cofactors necessary for this reaction are reduced pyridine nucleotides and a divalent cation (Mg^{++}, Mn^{++}, or Co^{++}). The reaction shows very little specificity for TPNH or DPNH, and in fact the differences shown in Figure 4 might be considered as due to differences in reduction potential of the two nucleotides. The squalene synthetase system of yeast was likewise found to be associated with cell particles and to need reduced pyridine nucleotides as coenzymes (4, 6). Spectrophotometric experiments (not reported here) have suggested a one-to-one stoichiometric relation between the amount of TPNH utilized and the amount of squalene formed. This conclusion is only provisional, however, because of the experimental difficulties encountered in measuring the allyl pyrophosphate dependent oxidation of small amounts of TPNH in the presence of microsomes.

The reductive step in squalene biosynthesis is somehow intimately connected with the tail-to-tail joining of two sesquiterpenoid intermediates, for in the absence of added TPNH or DPNH, all the C^{14} originally added as substrate can still be recovered as farnesyl pyrophosphate and free farnesol after 1 hour of incubation with washed microsomes. The possibility that a sulfhydryl group on the enzyme(s) is somehow involved in this reaction is suggested by some of the data presented in Table 8. Thus, PCMB and N-ethyl maleimide inhibited squalene synthesis from farnesyl pyrophosphate, and the PCMB inhibition could be reversed almost completely by addition of GSH. On the other hand, a third sulfhydryl inhibitor, iodoacetamide, was without effect on this reaction. The question of the participation of a sulfhydryl enzyme in this reaction hence cannot be clearly answered as yet, and is under further investigation.

The question of the existence of nerolidyl pyrophosphate as an enzymic product is of importance for the formulation of the detailed mechanism involved in the conversion of farnesyl pyrophosphate into squalene. Cornforth and Popják (29) have recently advanced an hypothesis for this mechanism which assumes the existence of an isomerase capable of transforming farnesyl pyrophosphate into nerolidyl pyrophosphate (11).

Condensation of 1 molecule of farnesyl pyrophosphate with 1 molecule of nerolidyl pyrophosphate (analogous to the condensation of dimethylallyl or geranyl pyrophosphate with isopentenyl pyrophosphate) then results in a triterpenoid compound which, by appropriate eliminations, stabilizes to dehydrosqualene, the reduction of which to squalene is presumed. It is stated, however, that the chemical hypothesis advanced does not require the existence of nerolidyl pyrophosphate per se, but rather that the same mechanism could apply for any nerolidyl derivative which would have properties similar to those expected of nerolidyl pyrophosphate. This hypothesis is in accord with all the known experimental facts of squalene biosynthesis, and it explains satisfactorily the findings of Rilling and Bloch (28), that during squalene biosynthesis from 5-D_2-mevalonate, 10 atoms of deuterium, out of a possible 12 atoms, were incorporated into squalene, and that the 2 atoms that disappeared were lost from the 2 central carbon atoms of squalene.

The results obtained in the present experiments unfortunately do not provide an unequivocal answer to the question of whether nerolidyl pyrophosphate exists as an enzymic reaction product or not. Given the instability of farnesyl pyrophosphate, and the facility with which this compound rearranges to nerolidol during nonenzymic hydrolysis, the small amounts of nerolidol observed after enzymic hydrolysis must be viewed with suspicion, as possibly arising from some slight nonenzymic hydrolysis. On the other hand, nerolidol has been observed in every case, no matter what method of enzymic hydrolysis was employed and despite all precautions against acid catalyzed hydrolysis and even after reductive cleavage with $LiAlH_4$. The fact that the percentage of nerolidol was small is virtually immaterial; Lynen *et al.* (6) reported, for example, that the equilibrium position of isopentenyl-pyrophosphate isomerase is such that an equilibrium mixture of the products contains 93 per cent dimethylallyl pyrophosphate and only 7 per cent of isopentenyl pyrophosphate. It is therefore felt that a definite answer to this question cannot as yet be given, and will depend on the development of a method for the separation of the several allyl pyrophosphates from one another. Only when nerolidyl pyrophosphate is separated by such a method, and the nerolidol shown to be optically active, will its existence as an enzymic reaction product be established conclusively. As mentioned above, however, uncertainty as to the existence of nerolidyl pyrophosphate need not materially affect the hypothesis of the mechanism of squalene synthesis given by Popják and Cornforth (31). The essential feature of this hypothesis is the condensation of farnesyl pyrophosphate with a nerolidyl derivative, and this latter could even be an enzyme-substrate

complex (or compound). Perhaps it is here that a sulfhydryl group participates in the process.

One other interesting feature of the liver enzyme system is the fact that when dialyzed soluble enzymes are added to the complete microsomal squalene synthesizing system, the yield of squalene from farnesyl pyrophosphate is reduced and conversion of the substrate to carboxylic acids occurs. The formation of "terpenoid" acids from mevalonate in liver enzyme incubations has been previously noted (32), *trans-trans*-farneosic acid being one of the acids identified. It is clear now from experiments carried out in collaboration with Dr. J. Christophe (to be published) that these acids arise by the action of soluble liver enzymes on the free allylic alcohols liberated from the pyrophosphates by the microsomal phosphatases. Unpublished studies indicate that these acids inhibit squalene synthesis, and the possibility exists that this system is involved in some way in the physiological regulation of endogenous cholesterol synthesis.

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