

STUDIES ON TERPENES:  
BIOSYNTHESIS AND OCCURRENCE

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THESIS



## ABSTRACT

### STUDIES ON TERPENES: BIOSYNTHESIS AND OCCURRENCE

By

Sandra Lee Baumann

This thesis reports the results of two projects: the first, a study of homomevalonate in enzyme systems; the second, investigations on the composition of beeswax.

Homomevalonate was employed as a substrate in enzyme systems known to catalyze the conversion of mevalonate to terpenes - namely, the enzymes prepared from yeast, rat liver, and pig liver. Failure to demonstrate incorporation in the presence of the rat liver and yeast enzymes was subsequently traced to the phosphate buffer employed in the incubation media. Pig liver enzymes prepared in Tris-HCl buffer catalyzed the conversion of HMVA to a compound postulated to be homofarnesol, in a medium which had a pH optimum of 7.2, a optimum ATP concentration of 7 mM and a stimulation by magnesium chloride that leveled at a concentration of 6.6 mM. A second labeled compound appearing at high ATP concentration or in the absence of NaF, is postulated to be an intermediate in homofarnesol biosynthesis.

Mass spectra and gas chromatograms of homomevalonolactone and its TMSi-derivative are reported along with information concerning the chromatographic behavior of other

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terpenes on GLC and TLC. Techniques are discussed for the resolution of problems relating to volatility and acid-sensitivity of prenols.

The study of beeswax was undertaken to determine the structure of the diols previously found in beeswax, the existence of prenols in wax, and the existence of a carbon skeleton similar to that of juvenile hormone in the hydrocarbons of beeswax.

The latter two investigations were negative while the assignment of structure to the diol fraction was tentative. Compositions of beeswax found in this investigation agreed with the literature reports.

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By

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## LIST OF ABBREVIATIONS

|       |                                                              |
|-------|--------------------------------------------------------------|
| ATP   | Adenosine triphosphate                                       |
| CoA   | Coenzyme A                                                   |
| GLC   | Gas-liquid chromatography                                    |
| HMVA  | Homomevalonic acid                                           |
| MVA   | Mevalonic acid                                               |
| NAD   | Nicotinamide adenine dinucleotide                            |
| NADH  | Nicotinamide adenine dinucleotide,<br>reduced form           |
| NADPH | Nicotinamide adenine dinucleotide<br>phosphate, reduced form |
| POPOP | 1,4-bis-[2-(5-Phenyloxazolyl)]-Benzene                       |
| PPO   | 2,5-Diphenyloxazole                                          |
| TLC   | Thin-layer chromatography                                    |
| TMSi  | Trimethylsilyl                                               |
| Tris  | Tris(hydroxymethyl)aminomethane                              |

# **STUDIES ON HOMOMEVALONATE**

## INTRODUCTION AND LITERATURE REVIEW

The search for a biological isoprenoid unit active in cholesterol biosynthesis began in 1954 with Rudney's observation that acetate gave rise to 3-hydroxy-3-methylglutaric acid (1). However, this compound was not the sought after intermediate since Coon and his coworkers showed that, in biological systems, 3-hydroxy-3-methylglutaric acid rapidly equilibrated with five other compounds (2). About two years later, Skeggs et al. found that the soluble residue from distiller's yeast contained a growth factor that could replace acetate in the incubation media of Lactobacillus acidophilus (3). The identification of this factor as mevalonolactone (3-hydroxy-3-methyl pentano-5-lactone) (4), coupled with its chemical similarity to 3-hydroxy-3-methyl glutaric acid, led Tavormina, Gibbs and Huff to test  $^{14}\text{C}$ -labeled mevalonate as an intermediate in cholesterol formation (5). Their isolation of radioactive sterol formed from the labeled precursor firmly established mevalonic acid (MVA) as an important precursor of the isoprene unit.

The link between acetate and mevalonate via acetoacetyl CoA and hydroxymethyl glutaryl CoA was subsequently established by the laboratories of Rudney (6) and Lynen (7).

After studying the incorporation of deuterium-labeled mevalonic acid, Bloch and coworkers theorized that terpenes were formed by the coupling of  $C_5$  subunits. These isoprene units were formed by concerted removal of the tertiary hydroxyl group and the carboxyl function from mevalonic acid (8) - a pathway supported by the previous observation by Tavormina and Gibbs that C-1 of mevalonate was eliminated as carbon dioxide (9).

The requirement of ATP and NADPH for the conversion of mevalonate to squalene by yeast extracts led Tchen to suspect a phosphorylated intermediate which he subsequently identified as the monophosphate of MVA (10). Lynen verified the structure of this intermediate as 5-phospho MVA (11) and added MVA-5-pyrophosphate and isopentenyl pyrophosphate to the growing list of intermediates (12). The description of the biosynthetic pathway up to the sesquiterpene stage was completed with the discovery of geranyl pyrophosphate (13) and farnesyl pyrophosphate (12). Liver enzymes, as shown by Popják et al., also utilized these intermediates (14). After further work on the isolation, properties, and biosynthesis of allyl pyrophosphates, Popják and Goodman were able to show the conversion of  $^{14}C$ -farnesyl pyrophosphate to squalene by a microsomal preparation from rat liver (15).

The availability of synthetic substrates labeled with deuterium or tritium enabled Cornforth and Popják to

study the stereochemical course of the reactions intervening between MVA-5-phosphate and squalene. Loss or retention of the isotopic label thus indicated the stereospecificity of the enzyme responsible for each conversion (16). Recent work from Popják's laboratory has dealt with the mechanism of the combination of isoprene units, with particular emphasis on the order of addition of substrates to the requisite enzymes (17, 18).

Still under investigation are the mechanisms for the conversion of farnesyl pyrophosphate to squalene and the subsequent cyclization to cholesterol. Using a yeast particulate fraction, Sofer and Rilling have discounted the role of nerolidyl pyrophosphate as an intermediate in the biosynthesis of squalene (19). This observation is clouded, however, by the fact that Popják et al. have found large amounts of nerolidol as a byproduct of the incubation of farnesyl pyrophosphate with yeast subcellular particles or liver microsomes. Furthermore, they isolated an intermediate between farnesyl pyrophosphate and squalene which was identified as the cyclic phosphoryl ester of squalene-10,11-glycol (20). Rilling previously reported the structure of this intermediate as a cyclopropane-containing C<sub>30</sub> pyrophosphate ester (21).

In elucidating the mechanism of the cyclization of squalene, the laboratories of van Tamelen and Corey simultaneously showed that rat liver microsomes can convert

2,3-oxidosqualene-<sup>18</sup>O into lanosterol-<sup>18</sup>O (22, 23). The former laboratory previously demonstrated the ability of rat liver homogenates to epoxidize squalene (24). The conversion of 2,3-oxidosqualene to lanosterol requires high ionic strength to dissociate the cyclase aggregate into active subunits (25). Evidently, the cyclization enzyme is relatively nonspecific, since the elimination of an entire isoprene unit from the non-oxidized end of squalene oxide did not prevent cyclization (26). Further investigation showed that the terminal monoepoxide of 2,3-dihydrosqualene and the diepoxide, 2,3,22,23-dioxidosqualene, are effective substrates for the cyclization enzymes, since incubation of these compounds with rat liver homogenates yielded the analogous sterol. Cyclization of the diepoxide proceeded at one-fourth the rate of 2,3-oxidosqualene (27). Corey's demonstration that 2,3-iminosqualene and decahydro-2,3-iminosqualene were potent inhibitors of the cyclase enzyme supports the view that cyclization is an electrophilic process triggered by protonation of the epoxide hydrogen and followed by nucleophilic attack of the double bonds on C-2. The observed inhibition is explained by the enhanced basicity of nitrogen over oxygen, coupled with the reduced electrophilicity of the aziridinium ion relative to a protonated epoxide adjacent to the double bond (28).

Another interesting development in the field of

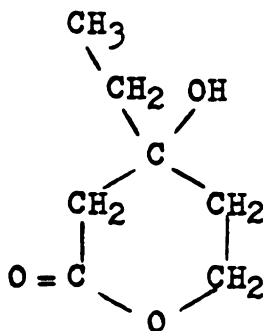
terpene biosynthesis has been the isolation of a radioactive mixture of partially saturated isoprenoid hydrocarbons from cell-free extracts of rat brain incubated with 2- $^{14}\text{C}$ -MVA. The existence of these hydrocarbons was suggested by the fact that the hexahydrochloride mother liquors remained radioactive after the removal of the squalene derivative. Further analysis of the nonsaponifiable fraction by thin-layer chromatography showed a number of radioactive spots that were less polar than squalene. After 24 hr. of incubation, a large number of these hydrocarbons was present, but one of them was already present after 2 hr. Extracts of adult rat brain which were incubated for 20 hr. with MVA and optimum cofactors also yielded a mixture of sterol-triterpene esters. However, only a small percentage of the sterol in this mixture was cholesterol. The structures of the other sterols remain to be studied (29).

Phospholipid has been implicated as an activator of terpene biosynthesis in a recent report from Chile (30). An investigation of a soluble enzyme system from Pinus radiata seedlings showed that phosphatidylethanolamine from plant sources markedly stimulated the transformation of MVA to prenyl pyrophosphates. Part of the activation was traced to the presence of unsaturated fatty acids since dipalmitoylphosphatidylethanolamine was ineffective. Exposure to air, which could oxidize double bonds, lessened

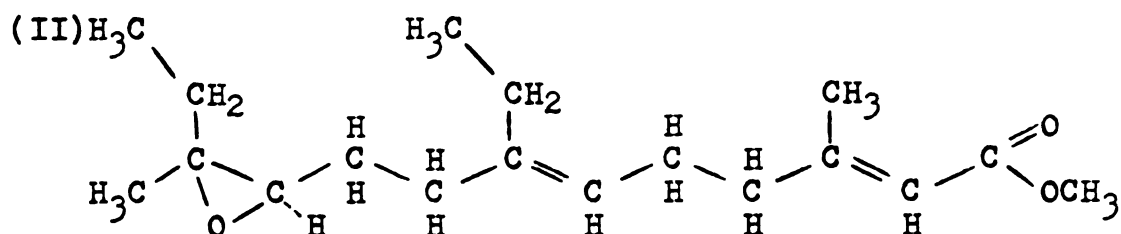
the activity of the phospholipid. Since lecithin was also ineffective, there was not absolute specificity for ethanolamine phosphatides. The phosphatides must be intact, however, since phospholipase treatment of phosphatidylethanolamine decreased its effectiveness. Detergents did not enhance MVA incorporation; therefore, the effect of phospholipid is not one of dispersion. The authors suggest that phosphatidylethanolamine acts on the conformation of enzymes or else facilitates the removal of water-insoluble products formed in this system. These latter two investigations provide a glimpse of the complexity and diversity of problems encountered in terpene biosynthesis.

The study of analogs of MVA began about the time of Tavormina's discovery when Tamura and Takai synthesized some homologs of MVA in an attempt to find a new antimetabolite for lactobacilli (31). Among these were  $\beta$ -ethyl- $\beta$ -hydroxy- $\delta$ -valerolactone, (I), hereafter referred to in the free acid form as homomevalonate (HMVA). This homolog did not replace acetate as a growth factor for Lactobacillus acidophilus, nor did its presence inhibit bacterial growth when mevalonate was added to the media (32).

(I)



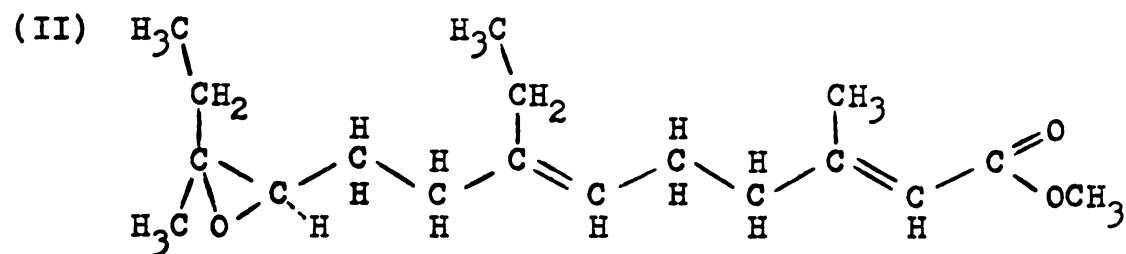
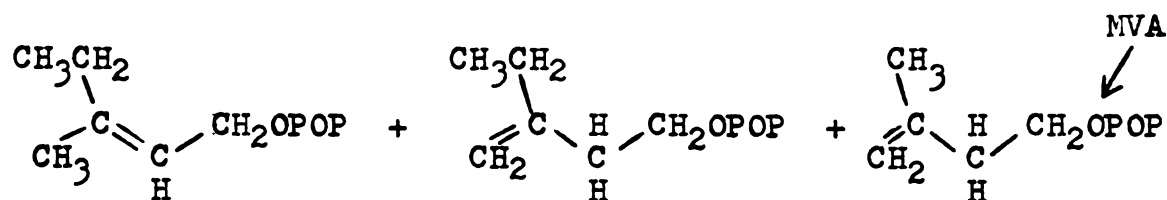
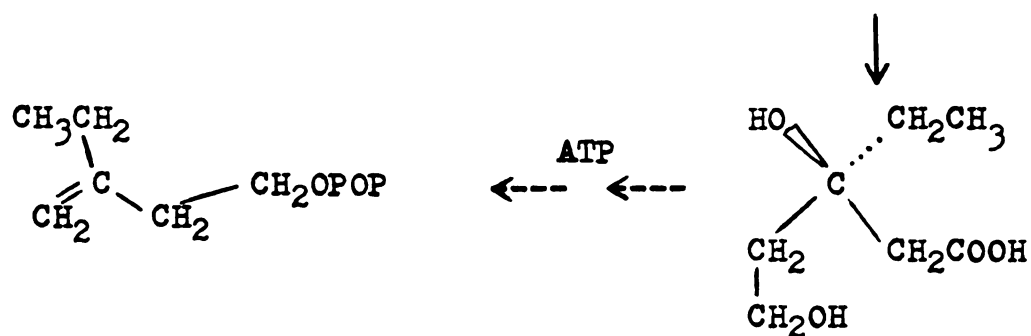
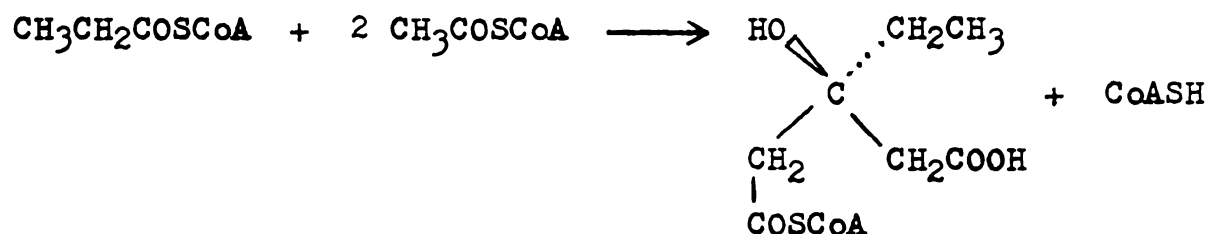
The impetus for further study of this mevalonate analog came from the elucidation, by Röllner, Dahm, Sweeley and Trost (33), of the structure of juvenile hormone as trans, trans, cis-10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate (II).



Although nothing is presently known about the mechanism of biosynthesis of this hormone, one possibility might be the combination of two  $C_6$  (homomevalonate) subunits and one  $C_5$  (mevalonate) subunit in a manner analogous to the biosynthesis of farnesol (see Figure 1). Since it is not at all certain whether insects are capable of assembling prenol chains, this study employed enzyme systems, from animal and yeast sources, which were known to utilize labeled mevalonate. The investigation was undertaken to determine whether such systems would accept homomevalonate (HMVA) as a substrate. In this way, information concerning the specificity of the enzymes involved in terpene biosynthesis could be gained.

Figure 1

## Possible Biosynthesis of Juvenile Hormone



## EXPERIMENTAL

### A. Materials

Chemicals for enzyme incubations were obtained from Mallinckrodt, St. Louis, Mo. All chemicals and solvents were reagent grade and used as supplied except where noted. Biuret, NADH, nicotinamide, ATP, Trisma base, alkaline phosphatase, NAD and squalene came from Sigma Chemical Company, St. Louis, Mo. DL-mevalonic acid-2-<sup>14</sup>C lactone with a specific activity of 13.4  $\mu\text{Ci}/\mu\text{mole}$  was purchased from Amersham Searle Corporation, Des Plaines, Ill. DL-homomevalonic acid-2-<sup>14</sup>C-lactone (1.6  $\mu\text{Ci}/\mu\text{mole}$ ) was the gift of William Gray and Dr. Theodore Cohen. Farnesol, nerolidol, and geraniol were obtained from K and K Laboratories, Inc., Hollywood, Calif.

The sources of chromatography supplies were: Kieselgel G and Silica Gel H - Brinkmann Instruments, Westbury, N.Y.; acid-washed Woelm alumina - Alupharm Chemicals, New Orleans, La.; Silicar TLC 7G - Scientific Products, Allen Park, Mich.; 250 micron pre-coated Chromar plates 7G - Scientific Glass Apparatus, Elk Grove Village, Ill.; Unisil - Clarkson Chemical Co., Williamsport, Pa. OV-1 and SE-30 column packings were purchased from Applied Science Laboratories, Inc., State College, Pa., while

hexamethyldisilazane and trimethylchlorosilane were obtained from the Anspec Co., Ann Arbor, Mich. Packard Instrument Company, Inc., Downers Grove, Illinois, was the source of POPOP and PPO for scintillation counting. Grooved chromatography plates for argentation thin-layers were the product of Kontes Glass Co., Wineland, N.J.

## B. Methods

### Protein Concentration

Routine determinations were done with biuret reagent from Sigma. Biuret (2.0 ml) was added to 1.0 ml. of buffer containing varied amounts of enzyme. After standing for fifteen minutes, the reaction mixture was read at 545 nm. on a Coleman Jr. spectrophotometer. Aliquots of a 20 mg./ml. solution of bovine serum albumin were used to establish the standard curve.

### Preparation of Silylation Reagent

Trimethylchlorosilane (2.0 ml.) and 4.0 ml. of hexamethyldisilazane were added to 10.0 ml. of dry pyridine. This silylation reagent was added to dry samples in the amount necessary to restore their original concentration. The samples were allowed to react for at least fifteen minutes before analysis.

### Liquid Scintillation Counting

Samples soluble in organic solvents were evaporated to dryness and counted in 10.0 ml. of DPO toluene (50 mg. POPOP and 4.0 g. PPO per liter of toluene). Silica gel samples were added directly to the scintillation cocktail; however, any iodine on the gel must evaporate before the addition of scintillator.

Aqueous samples were made up in 10.0 ml. of Bray's solution (10.5 g. PPO, 0.45 g. POPOP, 150 g. naphthalene, made to 1500 ml. with dioxane and then to 1800 ml. with glass distilled water).

All samples were counted for ten minutes in a Model LS-150 Beckman Scintillation Counter which had an external standard to determine quenching. A radioactive standard was counted to determine the efficiency of the instrument, and an aliquot of scintillation fluid was counted for background.

### Thin-Layer Monitoring

Thin-layer chromatograms 5 x 20 cm. on glass of 2 mm. thickness were scanned at one cm./min. on a Packard Model 7201 Radiochromatogram Scanner System. This system consists of a collector wire of 0.035 mm. diameter positioned axially in the center of a semicylindrical Geiger detector chamber which is opened to the chromatogram by a collimator. Dry helium passes continuously through the detector chamber. High voltage is applied between the

collector wire and the wall of the detector chamber. When a radioactive band passes under the collimator, the radiation flows into the gas. The ionization of the gas then releases electrons and positively charged ions. Since a potential is maintained across the gas, the electrons migrate to the collector wire. The signal pulses thus generated pass to a ratemeter which integrates them to indicate average counts per minute. This integrated signal is then recorded at the same speed as that of the chromatogram scan.

Solvent and dust were completely removed from the chromatograms prior to scan. A drop of radioactive marker was placed on each end of the plate for alignment of the radioactivity peaks. Most scans were done with a range of 300 cpm and a time constant of 30. Since the instrument had a  $^{14}\text{C}$  efficiency of twenty percent, a total of 1500 to 2000 cpm could be evaluated in this manner. The thickness of the glass on which the sorbent is coated is most important, since thicker glass will not pass successfully under the collimator.

#### Thin-Layer Chromatography

Silver nitrate plates were made by pouring a slurry of 30 g. Kieselgel G, 6 g. silver nitrate and 55 ml. glass distilled water onto a Kontes plate pre-ground with a 250 micron channel. The slurry was leveled with a stirring rod. After activation for one hour at  $100^{\circ}\text{C}$ ,

the plates were used immediately. Extra plates were kept in the dark but lost their separatory abilities after overnight storage. Neutral terpenes could be separated on these plates. Scanning with a UV light was used to visualize the spots on plates sprayed with 0.05% dichloro-fluorescein in 95% ethanol.

Pre-coated Chromar plates of 250 micron thickness or prepared Silicar 7G plates of 500 micron thickness were used to separate the terpenes resulting from enzyme incubation. A Desaga spreader was used to spread a slurry of 30 g. Silicar 7G in 55 ml. distilled water onto glass plates of 2 mm. thickness. These plates were dried for an hour at 100°C. Iodine vapors were used to visualize spots on the developed plates.

Prenyl pyrophosphates were evaluated on chromatograms prepared and developed according to Rilling (19).

Chromatography tanks were always used with a Whatman No. 1 filter paper liner and were allowed to equilibrate before use. Standards of the compounds of interest were always run on the same plate with experimental samples. Table 1 is a summary of the solvent systems used for various separations.

### Gas Chromatography

Analytical gas chromatography was done on an F & M 402 or a Perkin Elmer 900 chromatograph, both of which were equipped with hydrogen flame detectors. All liquid

Table 1

TLC Systems for Terpene Analysis

| Compounds                   | Plates                          | Solvent System                                               |
|-----------------------------|---------------------------------|--------------------------------------------------------------|
| Terpenes                    | AgNO <sub>3</sub> - Kieselgel G | Chloroform:acetone<br>(9:1)                                  |
| Prenols                     | Sillicar 7G                     | Benzene:ethyl acetate<br>(4:1)                               |
| Cholesterol and<br>squalene | Sillicar 7G                     | Chloroform:acetone<br>(9:1)<br>Hexane:diethyl ether<br>(4:1) |
| Pyrophosphates              | Buffered Silica gel H           | Chloroform:methanol:water<br>(60:40:9)                       |

phases were coated on 100-200 mesh acid-washed and silanized Gas Chrom S. The injection temperature was about 20° and the detector about 30° above the column temperature. Analyses were done on 3% OV-1 unless otherwise specified. Table 2 lists the temperatures necessary to analyze the compounds indicated.

### Mass Spectrometry

An LKB 9000 (LKB Produktor, Stockholm, Sweden) was used for mass spectral analyses. The instrument consists of a gas chromatograph and a single focusing 60° magnetic sector mass spectrometer, coupled directly with molecule separators of the Becker-Ryhage type. A 6 ft., 4 mm. i.d. coiled glass chromatographic column was used. The packing was 3% OV-1 on 100-200 mesh, acid-washed and silanized Gas Chrom S, with the column temperature adjusted to give convenient retention times for the compounds studied. The mass spectra were recorded at 70 eV with electron current of 60  $\mu$ amp. and accelerating voltage of 3500 V. The ion source temperature was 270°C.

### C. In Vitro Studies with Yeast Enzymes

#### Experiment 1 - Enzyme Purification

This experiment was designed to test the ability of Bloch's preparation of yeast enzymes (34) to synthesize sterol from MVA and to determine the necessity for dialysis and charcoal treatment of the enzyme. One milliliter of

Table 2  
GLC of Terpenes

| Compounds                            | Column Temperature<br>(°C) |
|--------------------------------------|----------------------------|
| Prenols                              |                            |
| Free                                 | 120                        |
| Trimethylsilyl derivatives           | 150                        |
| Homomevalonolactone, mevalonolactone |                            |
| Free                                 | 120                        |
| Trimethylsilyl derivatives           | 175*                       |
| Cholesterol and squalene             | 250                        |

\*This analysis was done on an Aerograph gas chromatograph equipped with an 8 ft. OV-17 column.

enzyme from each stage of preparation was added to incubation medium containing 1 mg. ATP, 1 mg. NADH, 1.5  $\mu$ moles magnesium chloride and 83,000 dpm of DL-2- $^{14}$ C-mevalonate. After three hours incubation, the samples were saponified with ethanolic potassium hydroxide and then extracted with four portions of 5 ml. diethyl ether. Aliquots of the ether solutions were counted and the remainder of the extracts evaporated to dryness. The residue was redissolved in 1 ml. of 2% ether in petroleum ether and applied to a 3 g. column of Woelm alumina, Brockman grade III. Squalene, prenols, sterol and precursor were respectively eluted by successive 25 ml. washes of 2% ether in petroleum ether, 12% ether in petroleum ether, 100% ether and methanol as per Stone and Hemming (35). Aliquots of these eluates were counted to determine the percent incorporation into each terpene component.

#### Experiment 2 - Phosphatase Effect

Persistently low incorporation of MVA was thought to result from the formation of ether-insoluble pyrophosphates. Therefore, the addition of phosphatase at the end of the incubation might liberate prenols and thus increase the observable incorporation of labeled MVA. The mevalonate tubes in this experiment contained 652,000 dpm while homo-mevalonolactone in chloroform solution was added at a level of 1,300,000 dpm. The chloroform was removed under a stream of nitrogen before cofactors were added. Both

dialyzed enzyme and crude homogenate were tested for activity while boiled enzyme was used in the blanks. Incubation, saponification, extraction and chromatography remained the same as Experiment 1. Following the initial incubation, one-half of the samples were treated with alkaline phosphatase under conditions reported by Bloch et al. (36). Carrier terpenes were added prior to extraction to ensure the recovery of radioactive products. The elution of labeled homomevalonolactone was also tested.

### Experiment 3 - Incubations with Free Homomevalonic Acid

Reflection on the lack of activity of mevalonolactone in enzyme systems (37) led to the conclusion that the lactone ring of homomevalonolactone must be opened if incorporation is to take place. Therefore, sodium hydroxide was added to convert the lactone to free acid. Incubation procedures remained the same and included treatment with phosphatase. Since column chromatography resulted in loss of radioactivity in the previous experiments, a new method of separating terpene components was developed. TLC seemed to be the logical choice; however, cholesterol was chromatographically similar to prenol. The terpenes did show a difference in the number of double bonds, thus, silver nitrate plates were tried and proved effective. Ether extracts of the incubated samples were separated on these plates and the appropriate bands counted in IP0 toluene. 1,800,000 dpm HMVA or 1,200,000 dpm MVA were added as substrates.

#### Experiment 4 - Evaluation of Labeled Products by GLC

The incubations for this experiment were the same as the previous experiment with the addition of samples containing unlabeled homomevalonate in the presence of radioactive mevalonate. The prenols were separated from neutral lipids (cholesterol and squalene) by the method of Goodman and Popják (15). These samples were sent to Dr. Bengt Samuelsson for GLC- $^{14}\text{C}$  analysis on a Barber-Coleman Radioactivity Monitor. When the samples were returned, the prenol fractions were analyzed on Silicar 7G plates. Cholesterol and squalene were also separated using these plates in a different solvent system.

#### Experiment 5 - Kinetics

This experiment was designed to determine whether homomevalonate competitively inhibited mevalonate incorporation. The incubation medium consisted of 1.0 ml. standard yeast enzyme, 1.0 mg. NADH, 1.0 mg. ATP, 1.5  $\mu$ moles magnesium chloride and a volume of 0.01 M phosphate buffer (pH 8) sufficient to make the final volume to 2.0 ml. Substrates were mevalonolactone and homomevalonolactone at a concentration of 10 mg./ml. in 0.01 M phosphate buffer, pH 8. At this pH, the lactone rings will open to form the free acids. Tube 1 contained cofactors but no enzyme; 2, cofactors, enzyme, no substrate; 3, cofactors, enzyme, 1.0 mg. MVA; 4, cofactors, enzyme, 1.0 mg. MVA, 2.0 mg. HMVA. The change in absorbance at

340 nm. was followed on a Gilford spectrophotometer maintained at 30°C. The experiment was repeated at the same conditions with twice the concentration of ATP and NADH.

#### D. In Vitro Studies with Rat Liver Homogenates

##### Experiment 1 - Effect of Acid for Prenol Liberation

The lack of definitive results from the yeast system led to the use of rat liver homogenates prepared by the method of Cornforth et al. (38). Incubations employed 3 mg. ATP, 2 mg. NADH, 2.5 ml. enzyme and 3,600,000 dpm HMVA or 2,400,000 dpm MVA. To ensure the complete conversion of HMVA to the free acid, the lactone was dissolved in 0.1 M phosphate buffer (pH 8) to give a solution which could be added directly to the incubation medium. After a 3 hour incubation at 37°C, the samples were made to 0.1 N HCl and carrier terpenes were added. Ether extracts of incubation tubes were analyzed on silica gel thin-layers impregnated with silver nitrate. The bands corresponding to squalene, cholesterol and prenols were scraped and counted in DP0 toluene.

##### Experiment 2 - HMVA as an Inhibitor of MVA Incorporation

This experiment differed from the previous one in that the method of Goodman and Popjak was used to extract prenols (15). Neutral lipids were removed from saponified samples by petroleum ether extraction after the addition of carrier cholesterol and squalene. Following neutral

lipid removal, the saponified samples were made to pH 1 and allowed to stand overnight. The next day, the pH was adjusted to 10; a mixture of standard prenol was added as carrier and the labeled prenols were extracted into petroleum ether. Silver nitrate plates were used to analyze the prenol fraction while silica gel thin-layers were used to separate cholesterol and squalene. Substrates employed in this experiment were 2,000,000 dpm MVA, 2,000,000 dpm HMVA, and the same amount of labeled MVA to which cold HMVA (1.0 mg.) in phosphate buffer had been added.

#### E. In Vitro Studies with Pig Liver Enzymes

##### Experiment 1 - First Incorporation of HMVA

Because of the difficulties encountered in isolating and characterizing cholesterol and squalene, the use of an enzyme system which synthesized prenyl pyrophosphates was indicated. A soluble system from pig liver, prepared by the method of Popják et al. (20), was employed since the preparation was stable for one year as the ammonium sulfate sludge and for one month as the dialyzed enzyme. Thus, the same enzyme preparation at a concentration of 55 mg./ml. could be used for all experiments.

The basic incubation medium consisted of 21  $\mu$ moles ATP, 15  $\mu$ moles magnesium chloride, 6  $\mu$ moles manganese chloride, 150  $\mu$ moles Tris-HCl buffer (pH 7.5) and 1.0 ml. dialyzed enzyme in a final volume of 3.0 ml. Blanks contained

1.0 ml. boiled enzyme. All substrates were dissolved in 0.3 M Tris buffer (pH 7.5) with 540,000 dpm mevalonate and 912,000 dpm homomevalonate. The following substrates or combinations were tested: labeled MVA; labeled HMVA; labeled MVA + 0.5 mg. unlabeled HMVA; labeled MVA + 1.0 mg. unlabeled HMVA; labeled HMVA + 1.0 mg. unlabeled MVA. The tubes were incubated, after being flushed with nitrogen, in screw-cap tubes on a shaking incubator at 37°C for 1½ hr. The reaction was terminated by boiling the tubes for 90 sec. After the tubes cooled in ice, 2.0 ml. 0.5 M Tris base and 3.0 mg. alkaline phosphatase (in 0.5 ml. 0.02 M  $\text{KHCO}_3$ ) were added; this reaction was allowed to stand overnight. The next day, 50 µl. carrier alcohols (3 mg./ml. each of geraniol, farnesol, nerolidol) were added and the tubes extracted with four portions of 5.0 ml. petroleum ether. The extracts were concentrated to approximately one milliliter on a rotary evaporator which was maintained at 40°C. This concentrate, combined with two 1.0 ml. washes, was transferred to an one dram vial and very carefully blown to dryness under nitrogen. Benzene (1.0 ml.) was added to each vial and 0.1 ml. removed for counting. Another aliquot (0.1 ml.) was streaked onto a Silicar 7G plate adjacent to a column of marker prenols. After being developed in benzene/ethyl acetate, the plate was scanned for radioactivity. Appropriate bands were removed and counted in DPO toluene.

### Experiment 2 - Effect of Buffer

This experiment employed the same basic incubation medium, including the same amount of radioactive substrate, as did the previous experiment. However, the pH of the buffer was varied from 7.2 to 7.8. Tris-HCl buffer (0.5 ml. of 0.3 M solution) was used except in one series of tubes where 0.3 M phosphate buffer, pH 7.5 was used. Suitable boiled enzyme blanks and mevalonate controls were employed. The methods for extraction and analysis of the samples were the same as Experiment 1.

### Experiment 3 - ATP Parameter

Standard conditions, including blanks and controls, were employed in this experiment except that 0.3 M Tris-HCl buffer, pH 7.2, was used, and the number of  $\mu$ moles of ATP varied from 7 to 25. Furthermore, 30  $\mu$ moles sodium fluoride were added to prevent breakdown of the pyrophosphates. The HMVA added was 1,400,000 dpm and the MVA, 1,190,000 dpm. Extractions remained the same.

### Experiment 4 - Magnesium Chloride Parameter

Conditions used in determining this parameter were the same as those of Experiment 3, including the amount of labeled substrates. The amount of ATP was 21  $\mu$ moles and magnesium chloride was varied from 5 to 20  $\mu$ moles.

## F. Effect of Evaporation

### Scintillation Counting

To test the effect of evaporating prenol samples for counting, 0.1 ml. each of benzene extracts of incubation media were placed in scintillation vials and carefully evaporated to dryness under nitrogen. Then 10 ml. DPO toluene were added. Similarly, 0.1 ml. of the same extracts dissolved in toluene was added directly to scintillation cocktail.

### Thin-Layer Chromatography

A thin-layer plate which contained 0.1 ml. of a prenol mixture extracted from a homomevalonate incubation was scanned for radioactivity and allowed to stand overnight. The next day, the plate was re-scanned under the same conditions.

## G. Effect of Acid Treatment on Terpenes

Part of an ether extract (200  $\mu$ l.) containing labeled farnesol from a mevalonate incubation was plated onto Silicar 7G for TLC in benzene/ethyl acetate. After the plate was scanned for radioactivity, the band corresponding to farnesol was removed and eluted with benzene followed by diethyl ether. The solvent was concentrated to approximately 1 ml. on a rotary evaporator and added to 2.0 ml. 1.0 N HCl in methanol. After standing overnight, the solution was neutralized with

silver carbonate and the precipitate removed by centrifugation. The methanolic solution was concentrated under a stream of nitrogen and streaked onto a Silicar 7G plate. After development in benzene/ethyl acetate, the plate was scanned for radioactivity and exposed to iodine vapors.

#### H. Conversion of Prenyl Pyrophosphates to Sterol

Prenyl pyrophosphates were prepared from 1,080,000 dpm MVA and 1,824,000 dpm HMVA in incubations with 20  $\mu$ moles magnesium chloride, 6  $\mu$ moles manganese chloride, 120  $\mu$ moles Tris buffer (pH 7.2), 21  $\mu$ moles ATP, 30  $\mu$ moles sodium fluoride and 1.0 ml. pig liver enzymes. The pyrophosphate mixture from each substrate was extracted from the incubation medium with butanol by the method of Sofer and Billing (19). After the butanol was removed, the pyrophosphate residue was dissolved in 0.02 M  $\text{KHCO}_3$  and lyophilized overnight.

This pyrophosphate preparation was used as a substrate for a microsomal enzyme system prepared from rat liver by the method of Popjak (15). The incubation medium consisted of 2 mg. plasma albumin, 10  $\mu$ moles NaF, 3  $\mu$ moles NADH, 30  $\mu$ moles nicotinamide, 60  $\mu$ moles phosphate buffer (pH 7.4), 5  $\mu$ moles magnesium chloride and 0.1 ml. microsomal enzyme (0.4 mg.) added directly to the lyophilization tubes. Samples were mixed, flushed with nitrogen for 90 sec., sealed and incubated at 37°C for one hour. After

the samples cooled, 300  $\mu$ g. squalene were added and the solutions adjusted to pH 10 by addition of KOH. Four portions of 5 ml. petroleum ether were used to extract radioactive product. The extracts were concentrated and transferred to one dram vials. After the solvent was carefully evaporated, 1.0 ml. benzene was added to the vials. A tenth of this volume was taken for counting; then 0.2 ml. from HMVA samples and 0.1 ml. from MVA samples were developed on Silicar plates in benzene/ethyl acetate. Similar samples were also developed in diethyl ether/hexane. Bands corresponding to sterol and squalene were scraped and counted in DPO toluene.

## RESULTS

### A. Thin-Layer Chromatography of Terpenes

A summary of terpene mobilities in various solvent systems is found in Table 3. When a buffer solution of either labeled mevalonate or labeled homomevalonate was streaked onto Silicar plates and developed in either the standard prenol system or the standard sterol system, all the radioactivity, as determined by scanning, remained at the origin. Thus, any radioactive precursor that might have been extracted from incubation medium would not have interfered with the analysis of labeled products.

### B. GLC of Terpenes

Figure 2 shows a gas chromatogram of homomevalonolactone on 3% OV-1 at 120°C. At this temperature, the lactone had a retention time of 7.5 minutes and gave a characteristic tailing peak. The small amount of impurity was the dehydrated lactone which could have been formed during the GLC analysis according to a report by Gray (39).

With the addition of an internal standard, both mevalonolactone and homomevalonolactone can be quantitated by GLC analysis. After a number of compounds were tested, tetradecanol at a concentration of 1.0 mg./ml. was found

Table 3  
Analysis of Terpenes by TLC

| Solvent System and Sorbent                             | Compound         | R <sub>f</sub> |
|--------------------------------------------------------|------------------|----------------|
| Chloroform:acetone (9:1)<br>Silver nitrate-Kieselgel G | Cholesterol      | 0.70           |
|                                                        | Squalene         | 0.27           |
|                                                        | Prenols          | 0.09           |
| *Benzene:ethyl acetate (4:1)<br>Silicar 7G             | Nerolidol        | 0.60           |
|                                                        | Farnesol, band 1 | 0.50           |
|                                                        | Farnesol, band 2 | 0.47           |
|                                                        | Geraniol         | 0.39           |
| Hexane:diethyl ether (9:1)<br>Silicar 7G               | Squalene         | 0.77           |
|                                                        | Lanosterol       | 0.19           |
|                                                        | Cholesterol      | 0.10           |
|                                                        | Farnesol         | 0.05           |
|                                                        | Nerolidol        | 0.13           |
| **Chloroform:acetone (9:1)<br>Silicar 7G               | Squalene         | 0.75           |
|                                                        | Farnesol         | 0.66           |
|                                                        | Cholesterol      | 0.56           |
|                                                        | Lanosterol       | 0.63           |

\*Standard prenol separation

\*\*Standard sterol separation

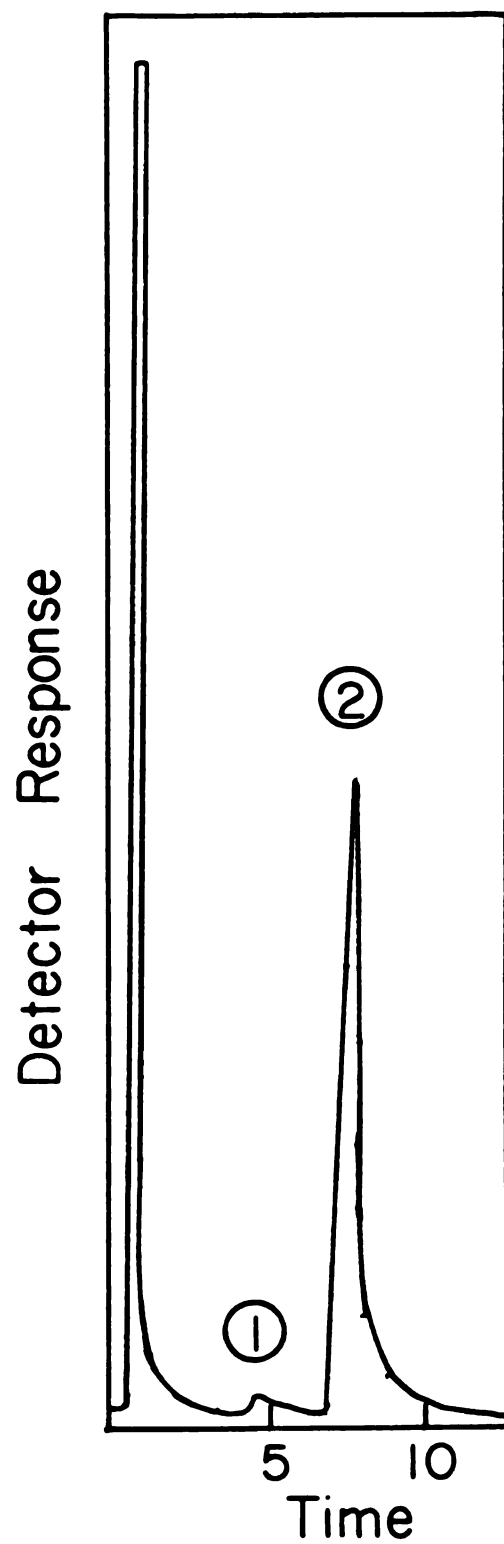


Figure 2. GLC of homomevalonolactone. [3% OV-1, 120°C]. Peak I is a small impurity; peak 2 is the lactone.

to have the requisite properties for use as an internal standard. The trimethylsilyl derivative of tetradecanol served a similar function in the analysis of the silylated lactones as shown in Figure 3. At 175°C on an eight-foot column of OV-17, these compounds had the following retention times: TMSi-mevalonolactone, 8.1 minutes; TMSi-homomevalonolactone, 12.0 minutes; TMSi-tetradecanol, 16.8 minutes. Under the same conditions, the free compounds had retention times of 9.3 minutes for mevalonolactone, 14.9 minutes for homomevalonolactone and 18.9 minutes for tetradecanol. The amount of each lactone was determined from its peak area relative to that of tetradecanol.

As shown in Figure 4, free prenols were separated at 120°C on 3% OV-1. Since the prenols were very volatile, the trimethylsilyl derivatives were prepared when quantitation was desired. The gas chromatogram of these derivatives at 150°C on 3% OV-1 (Figure 5) showed less tailing of peaks than was observed with the free prenols.

Cholesterol and squalene had respective retention times of 13.4 minutes and 6.2 minutes when they were analyzed at 250°C on 3% OV-1 (Figure 6).

### C. Mass Spectra

The mass spectrum of free homomevalonolactone is shown in Figure 7. A molecular ion was observed at  $m/e$  144 and large fragment ions at  $m/e$  126 ( $M-18$ ),  $m/e$  115

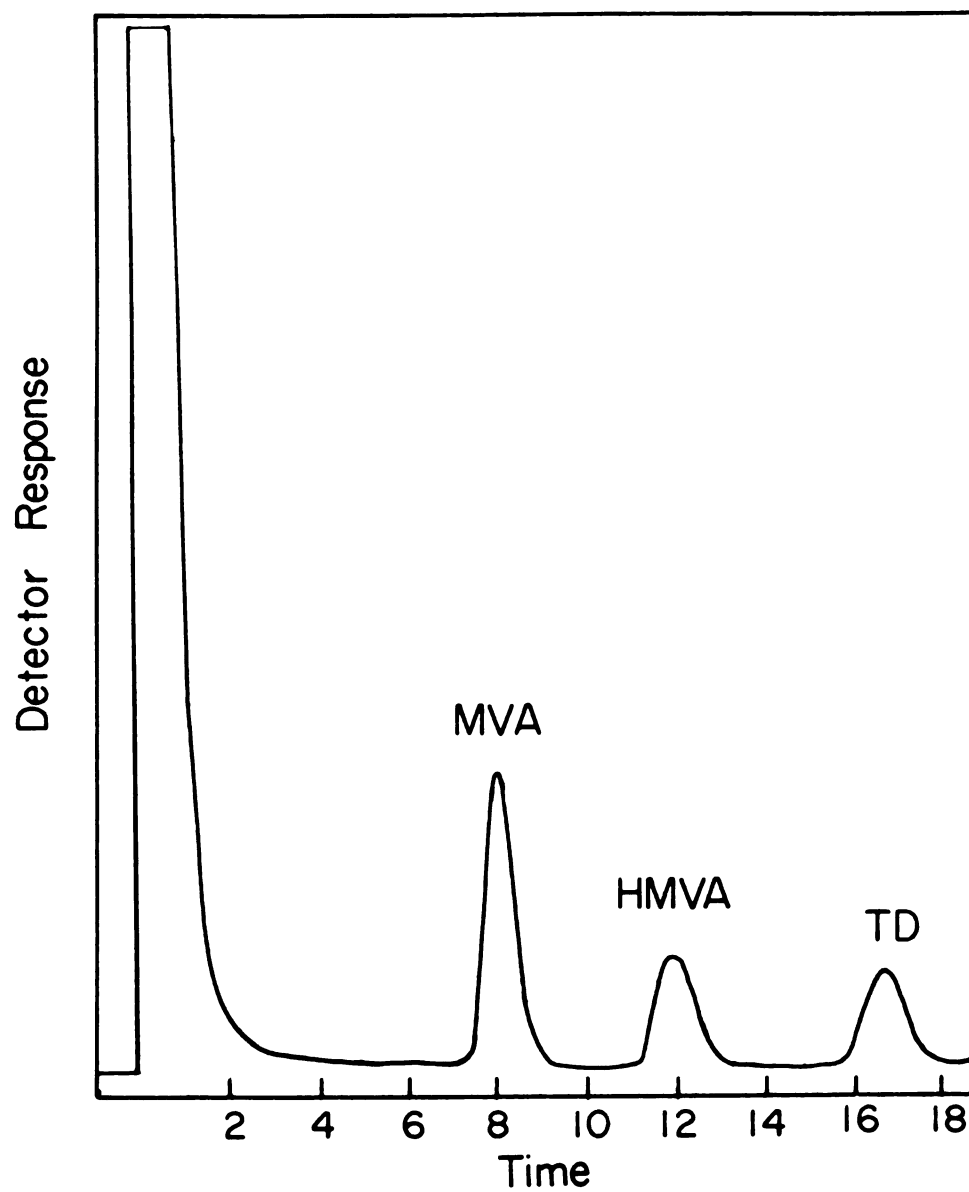


Figure 3. GLC of TMSi-homomevalonolactone (HMVA), TMSi-mevalonolactone (MVA), TMSi-tetradecanol (TD). [3% OV-17, 175°C].

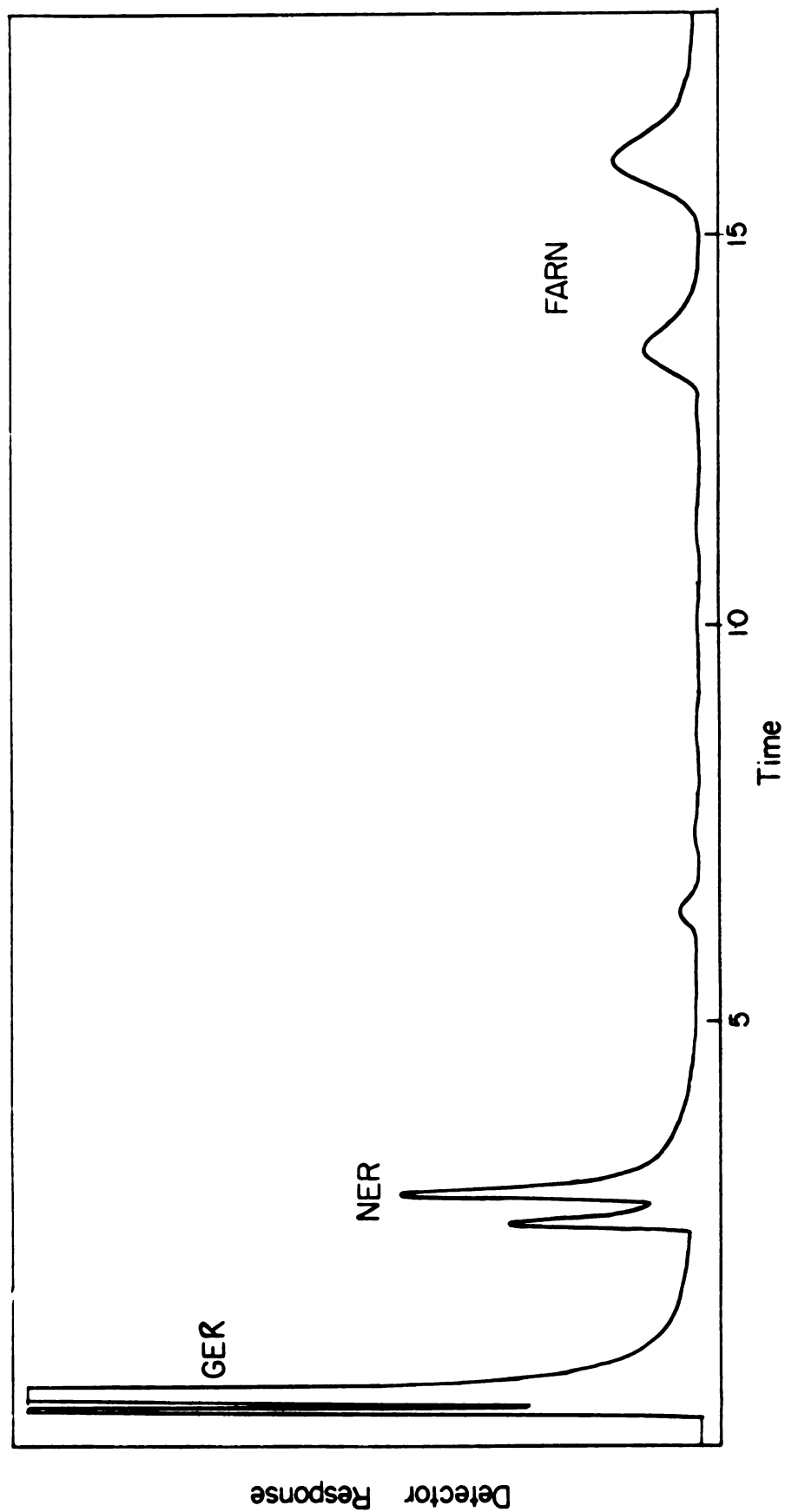


Figure 4. GLC of free prenols: geraniol (GER), nerolidol (NER), farnesol (FARN).  
[3% OV-1, 120°C]

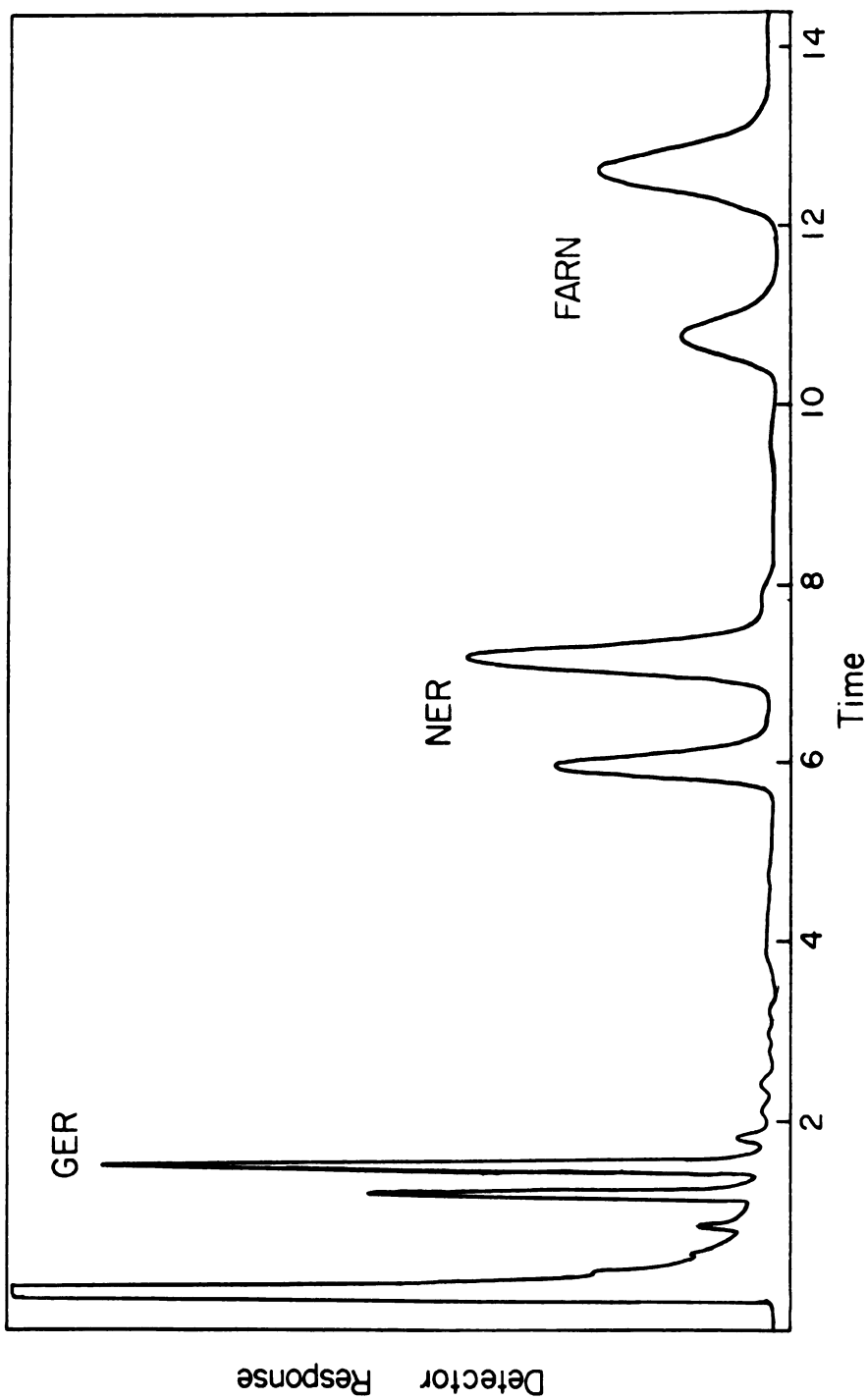


Figure 5. GLC of TMSi-prenols: geraniol (GER), nerolidol (NER), farnesol (FARN).  
[3% OV-1, 150°C]

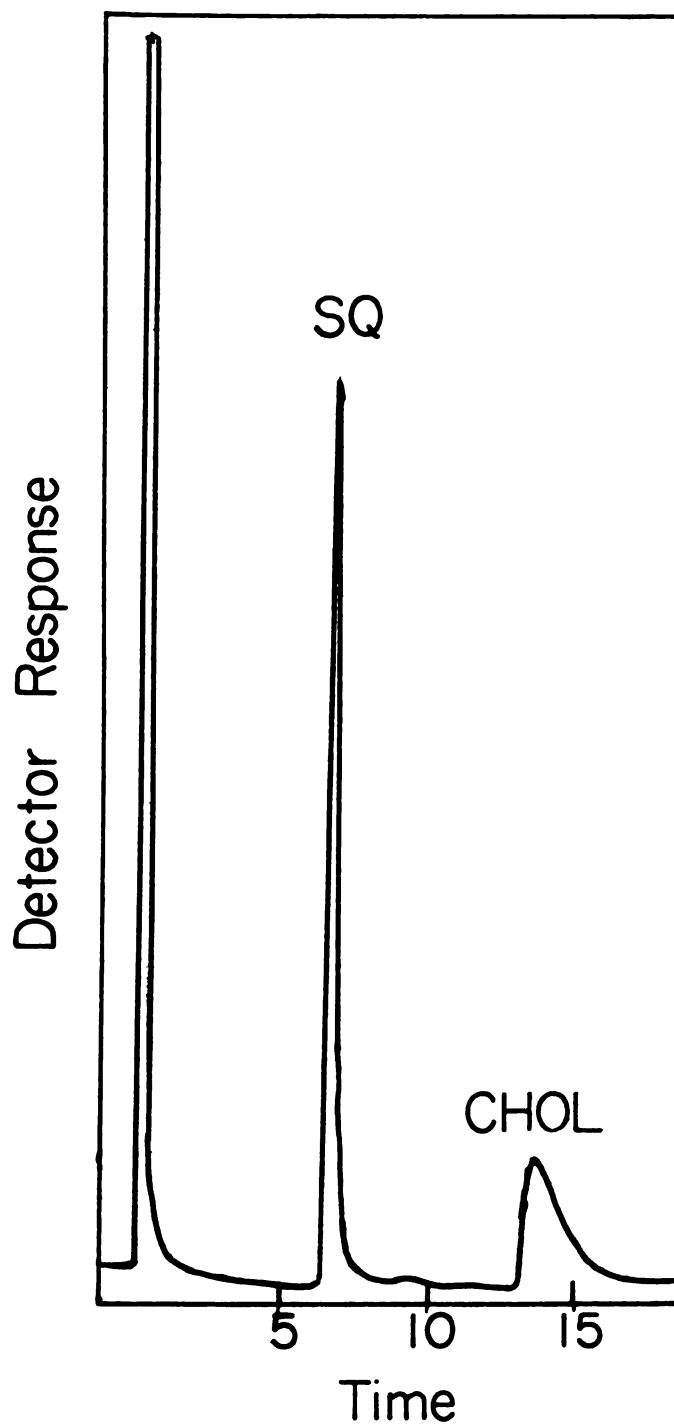


Figure 6. GLC of cholesterol (CHOL) and squalene (SQ).  
[3% OV-1, 250°C].

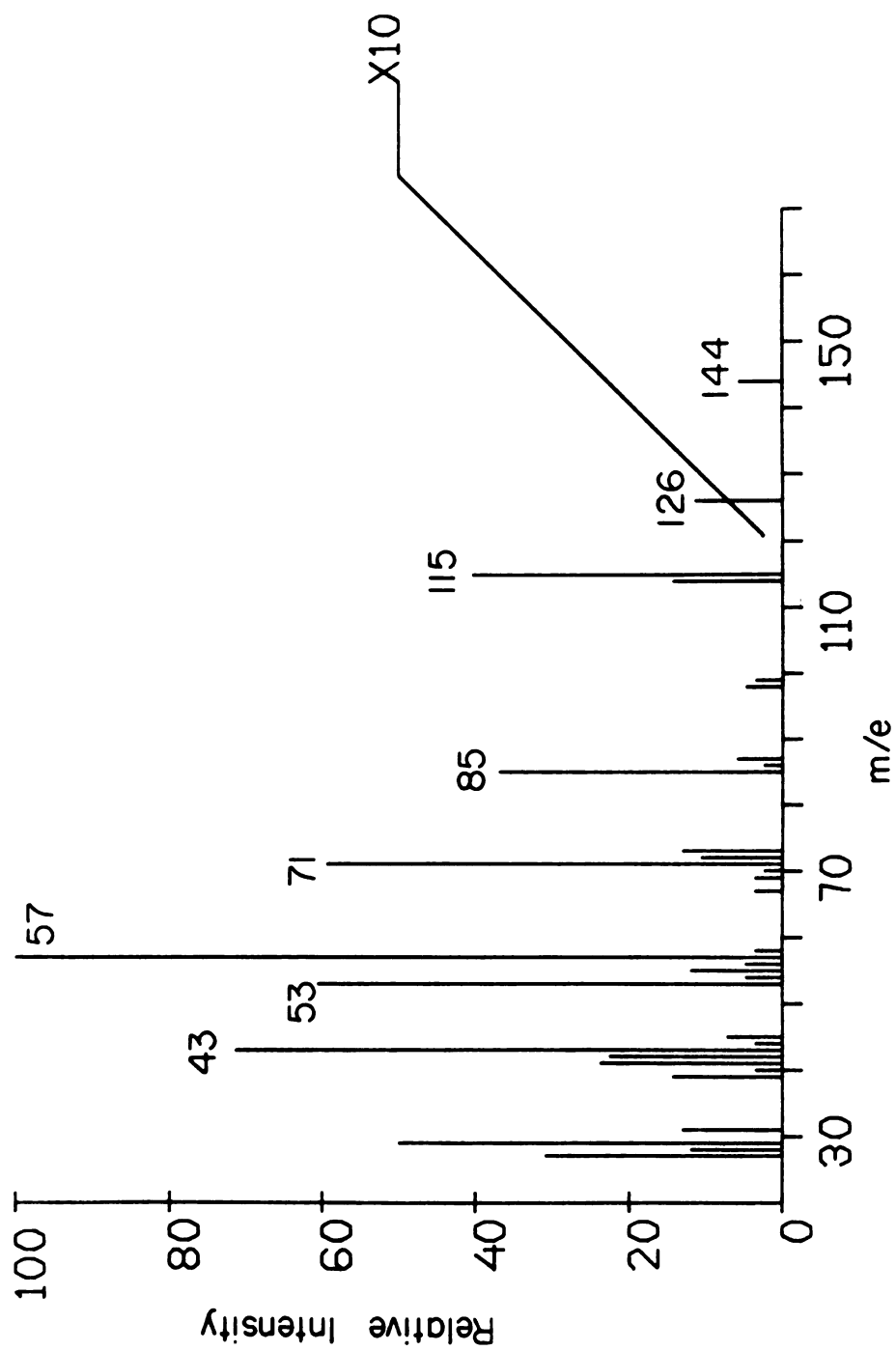


Figure 7. Mass spectrum of homomevalonolactone.

(M-29) and m/e 85 (M-59) helped to confirm the structure of this synthetic compound. The mass spectrum of TMSi-homomevalonolactone (Figure 8) showed the expected peaks at m/e 201 (M-15), m/e 187 (M-27), m/e 159 (M-29-28) and m/e 73  $[\text{Si}(\text{CH}_3)_3]^+$ .

#### D. In Vitro Studies with Yeast Systems

##### Experiment 1 - Enzyme Purification

Table 4 summarizes the incorporation of mevalonate into ether-soluble compounds by yeast enzyme preparations at various stages of purification. Dialysis of the enzyme preparation resulted in the greatest increase in mevalonate utilization; the charcoal treatment was therefore discarded since it removed necessary cofactors.

##### Experiment 2 - Effect of Phosphatase Treatment

The previous experiment showed relatively low incorporation of labeled mevalonate into terpene. This could be attributed to the formation of ether-insoluble prenyl pyrophosphates which were not hydrolyzed during saponification. Therefore, an incubation was set up to determine whether alkaline phosphatase would release more radioactivity into the ether extracts. Table 5 shows that samples incubated with MVA and treated with phosphatase had a 10-fold increase in the ether-extractable radioactivity. The major portion of this radioactivity was found in the sterol fraction when the substrate was MVA, and in the precursor fraction when



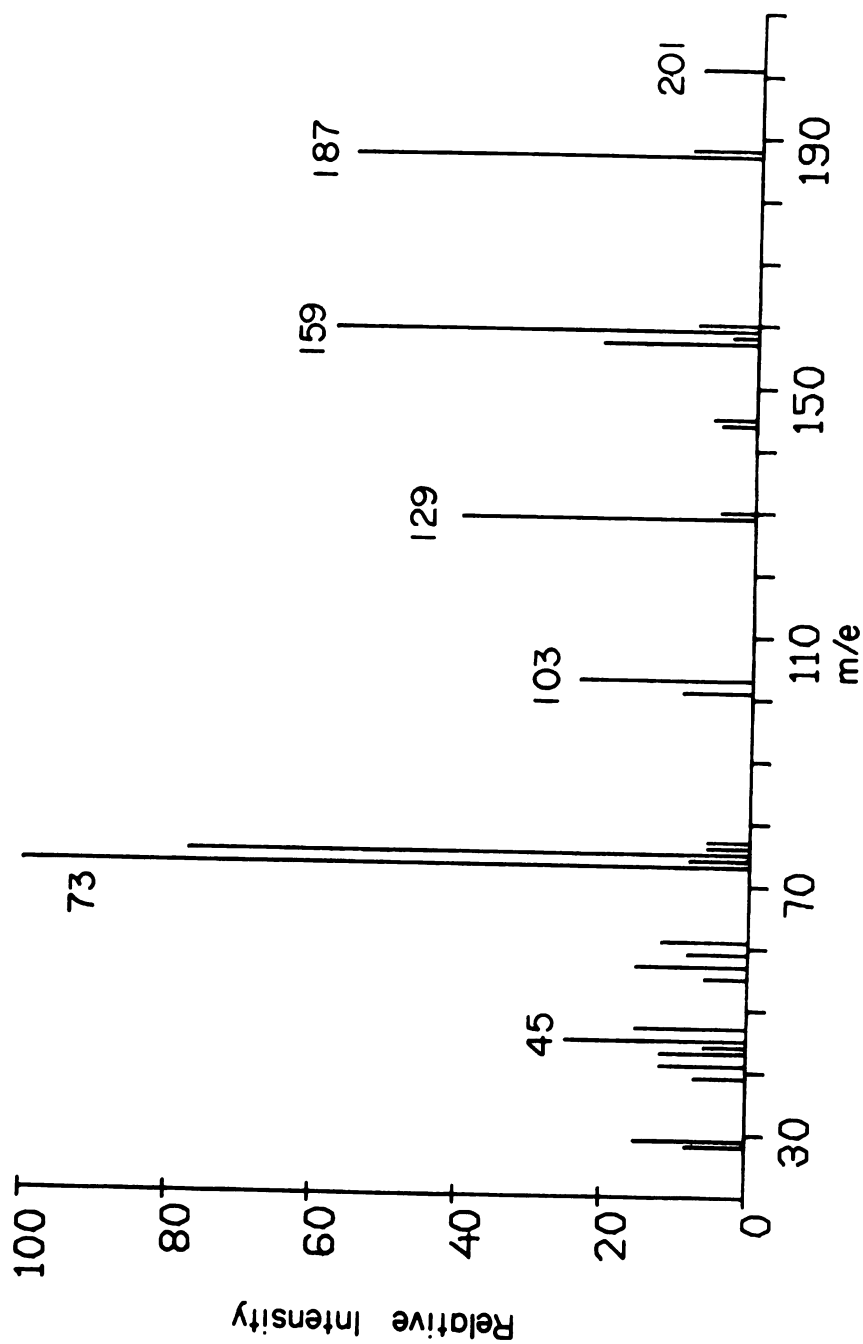


Figure 8. Mass spectrum of TMSi-homomevalonolactone.

Table 4  
Enzyme Purification in MVA Incorporation

| Sample               | % Incorp. into Ether Extract <sup>a</sup> | Squalene | Prenol | Sterol |
|----------------------|-------------------------------------------|----------|--------|--------|
| Enzyme-no-dialysis   | 1.4                                       | 0.45     | 0.21   | 0.14   |
| Enzyme-post-dialysis | 51.9                                      | 1.3      | 0.15   | 5.36   |
| Enzyme-post-charcoal | 15.3                                      | 0.28     | 0.11   | 6.93   |

<sup>a</sup>% incorporation based on half of the 83,000 dpm MVA added.

Table 5  
Effect of Phosphatase Treatment

| Tube No. | Enzyme    | Substrate           | % Incorp. into Ether<br>Extract <sup>a</sup> |
|----------|-----------|---------------------|----------------------------------------------|
| 1        | boiled    | homomevalonolactone | 0                                            |
| 2        | dialyzed  | homomevalonolactone | 0.5                                          |
| 3        | dialyzed  | homomevalonolactone | 0.6                                          |
| 4        | crude     | homomevalonolactone | 0.2                                          |
| 5        | dialyzed  | mevalonate          | 4.2                                          |
| 6        | dialyzed  | mevalonate          | 3.4                                          |
| 7        | dialyzed* | homomevalonolactone | 9.5                                          |
| 8        | dialyzed* | homomevalonolactone | 6.1                                          |
| 9        | crude*    | homomevalonolactone | 16.5                                         |
| 10       | dialyzed* | mevalonate          | 40.0                                         |
| 11       | dialyzed* | mevalonate          | 45.0                                         |

\*Incubation media treated with phosphatase.

<sup>a</sup>% incorporation based on one-half the radioactivity added: MVA, 652,000 dpm; homomevalonolactone, 1,300,000 dpm.

the substrate was homomevalonolactone. This difference in incorporation might be attributed to the inactivity of lactones in enzyme systems.

Alumina chromatography of labeled homomevalonolactone resulted in an elution of 1% of the counts in the 100% ether (sterol) fraction with the remainder of the radioactivity in the methanol wash. Therefore, the three terpene fractions were uncontaminated by precursor. This is further substantiated by the lack of counts in the ether extract of the boiled enzyme blank.

#### Experiment 3 - Incubations with Homomevalonic Acid

This experiment, utilizing the free acid liberated from homomevalonolactone by treatment with base, should allow more valid conclusions to be drawn concerning the metabolic activity of this analog. Table 6 shows little incorporation with either HMVA or MVA substrates. The enzyme preparation might have been inactive; however, this is unlikely since the enzyme was prepared by the same method that gave activity in the previous experiments. Another possible explanation is that the incubation media were not saponified; therefore, incorporation of substrate into sterol esters would not be detected. However, large amounts of such esters were not expected.

#### Experiment 4 - Evaluation of Products by GLC Radioactivity Monitoring

Of the samples sent to Dr. Samuelsson, the ones

Table 6  
Incorporation of Homomevalonic Acid

| Tube       | % Incorporation of $^{14}\text{C}^a$ |          |        |
|------------|--------------------------------------|----------|--------|
|            | Prenol                               | Squalene | Sterol |
| HMVA blank | 0.009                                | 0.011    | 0.003  |
| HMVA test  | 0.328                                | 0.006    | 0.010  |
| HMVA test  | 0.113                                | 0.007    | 0.005  |
| MVA blank  | 0.022                                | 0.001    | 0.022  |
| MVA test   | 0.033                                | 0.003    | 0.002  |
| MVA test   | 0.012                                | 0.004    | 0.003  |

<sup>a</sup>Based on 50% of the radioactivity added: 1,800,000 dpm HMVA and 1,200,000 dpm MVA.

resulting from HMVA incubations had too few counts to be assayed. The prenol series from MVA incubations showed radioactivity in farnesol and nerolidol, whereas the neutral lipid series showed radioactivity in a peak at the solvent front and in the peak corresponding to squalene, but none in the cholesterol peak. Analysis of the neutral lipid samples by TLC revealed two bands of radioactivity: one at an  $R_f$  corresponding to squalene and one at an  $R_f$  that seemed to correspond to cholesterol. Closer inspection of the thin-layer chromatogram revealed that the second radioactive component had an  $R_f$  slightly higher than that of cholesterol and might therefore be farnesol. Since microsomes were not removed from the enzyme preparation, microsomal phosphatase could have attacked the farnesyl pyrophosphate synthesized, thereby releasing labeled farnesol. The presence of liberated farnesol in the neutral lipid samples could also account for the radioactivity that Samuelsson found in the solvent peak, since the temperature employed in the analysis of cholesterol and squalene was  $130^{\circ}$  higher than that employed in the routine analysis of farnesol.

#### Experiment 5 - Kinetics

The attempt to determine the kinetics of HMVA in the presence of MVA failed. In the first experiment, the absorbance of tube 1 remained constant while the absorbances of tubes 2, 3 and 4 declined at the same rate. The absor-

bances of all the tubes in the repeat experiment remained the same.

#### E. In Vitro Studies with Rat Liver Homogenates

##### Experiment 1 - Effect of Acid on Prenol Liberation

Table 7 shows that acid treatment of the incubation medium to release prenol from the pyrophosphates resulted in a boiled enzyme blank with a large amount of radioactivity. TLC analysis of the ether extract showed that most of the radioactivity was in the prenol fraction. The prenol fraction of the remaining samples also had high counts.

When 2-<sup>14</sup>C-homomevalonolactone was analyzed on the silver nitrate plates employed in this assay, the majority of the counts were similarly found in a band with an  $R_f$  corresponding to that of prenol. On the other hand, a chromatogram of the free homomevalonic acid used as precursor revealed radioactivity only at the origin.

This assay would not detect radioactivity in the sterol ester fraction since bands corresponding to sterol ester were not scraped.

##### Experiment 2 - HMVA as an Inhibitor of MVA Incorporation

Since HMVA was not incorporated into terpene during any of the experiments previously described, a test was made of its activity as an inhibitor of MVA utilization. The amount of MVA present was 9.0  $\mu$ g. (calculated from a

Table 7  
Effect of Acid on Terpene Extraction

| Substrate  | Enzyme | % Incorporation <sup>a</sup> |          |        |
|------------|--------|------------------------------|----------|--------|
|            |        | Prenol                       | Squalene | Sterol |
| homo       | boiled | 28                           | 0.8      | 0.3    |
| homo       | active | 20                           | 0.4      | 0.2    |
| homo       | active | 5                            | 0.1      | 0.1    |
| mevalonate | active | 4                            | 0.8      | 1.0    |
| mevalonate | active | 4                            | 1.0      | 1.6    |

<sup>a</sup>Based on 50% of the radioactivity added: homo-mevalonate (homo), 3,600,000 dpm; mevalonate, 2,400,000 dpm.

specific activity of 103  $\mu\text{Ci./mg.}$ ), while homomevalonate was added in a 100-fold excess (1.0 mg.). This amount of HMVA should swamp the MVA and result in a greater inhibition than the observed effect (20% inhibition) which is shown in Table 8.

#### F. In Vitro Studies with Pig Liver Enzymes

##### Experiment 1 - First Demonstration of Homomevalonate Incorporation

This experiment, utilizing a pig liver enzyme system prepared in Tris-HCl buffer, demonstrated the incorporation of homomevalonate for the first time. The results are summarized in Table 9.

A petroleum ether extract of the HMVA boiled enzyme blank contained a large amount of radioactivity which remained at the origin of a thin-layer chromatogram developed in benzene/ethyl acetate. A buffer solution of  $^{14}\text{C}$ -homomevalonate chromatographed under the same conditions also showed a single radioactive peak at the origin. Extracts of incubation medium containing HMVA and active enzyme gave two bands of radioactivity: one at an  $R_f$  of 0.51 which was slightly higher than that for the upper band of farnesol ( $R_f$  0.47), and another at  $R_f$  0.31 which was slightly lower than that of geraniol ( $R_f$  0.35).

Mevalonate incubations (tubes 5 to 10) gave rise to a single radioactive band at an  $R_f$  corresponding to that

Table 8  
Further Studies of HMVA in Rat Liver Systems

| Tube No. | Substrate <sup>b</sup> | Enzyme | % Incorporation <sup>a</sup> |          |        |
|----------|------------------------|--------|------------------------------|----------|--------|
|          |                        |        | Sterol                       | Squalene | Prenol |
| 1        | H*                     | boiled | 0.005                        | 0.001    | 0.002  |
| 2        | H*                     | active | 0.004                        | 0.001    | 0.010  |
| 3        | H*                     | active | 0.002                        | 0        | 0.046  |
| 4        | M*                     | boiled | 0                            | 0        | 0.119  |
| 5        | M*                     | active | 1.45                         | 0.32     | 4.8    |
| 6        | M*                     | active | 1.53                         | 0.37     | 5.0    |
| 7        | M* + H                 | active | 1.31                         | 0.34     | 4.5    |
| 8        | M* + H                 | active | 0.86                         | 0.39     | 3.9    |

<sup>a</sup>Based on 50% of the radioactivity added: 2,000,000 dpm each of mevalonate and homomevalonate.

<sup>b</sup>M\* is 2-<sup>14</sup>C-mevalonate; H\* is 2-<sup>14</sup>C-homomevalonate; H is 1.0 mg. unlabeled homomevalonate.

Table 9  
Initial Studies on Pig Liver Enzyme System

| No. | Sample <sup>b</sup> | % Incorporation <sup>a</sup> |      |                            |      |
|-----|---------------------|------------------------------|------|----------------------------|------|
|     |                     | Ether Extract                |      | Farnesol Band <sup>c</sup> |      |
|     |                     | Avg                          |      | Avg                        |      |
| 1   | H* blank            | 1.13                         |      | ---                        |      |
| 2   | H*                  | 4.00                         |      | 1.78                       |      |
| 3   | H*                  | 2.40                         | 3.20 | 0.97                       | 1.38 |
| 4   | M* blank            | 0.12                         |      | ---                        |      |
| 5   | M*                  | 52.3                         |      | 48.2                       |      |
| 6   | M*                  | 64.0                         | 58.2 | 57.0                       | 52.5 |
| 7   | M* + H              | 57.6                         |      | 54.0                       |      |
| 8   | M* + H              | 64.8                         | 61.2 | 54.0                       | 54.0 |
| 9   | M* + 2 H            | 54.1                         |      | 48.5                       |      |
| 10  | M* + 2 H            | 57.0                         | 55.5 | 52.0                       | 50.2 |
| 11  | H* + M              | 0.63                         |      | 0.26                       |      |
| 12  | H* + M              | 0.36                         | 0.50 | 0.19                       | 0.22 |

<sup>a</sup>Based on 50% of the radioactivity added: 540,000 dpm mevalonate and 912,000 dpm homomevalonate.

<sup>b</sup>H\*, labeled homomevalonate; M\*, labeled mevalonate; H, 0.5 mg. unlabeled homomevalonate; 2 H, 1.0 mg. unlabeled homomevalonate; M, 1.0 mg. unlabeled mevalonate.

<sup>c</sup>This band is "homofarnesol" when homomevalonate is the substrate.

of farnesol. If tube 5 was low because of a pipetting error, tubes 7 through 10 might be construed to show a slight inhibition of MVA incorporation when HMVA was added to swamp. Thin-layer chromatograms of the ether extracts of these samples showed slightly decreasing amounts of radioactivity in the farnesol band. A much greater inhibition was expected, however, since the amount of unlabeled HMVA added (0.5 mg. or 1.0 mg.) was a 200 or 400-fold excess of the amount of  $^{14}\text{C}$ -MVA (calculated to be 2.4  $\mu\text{g.}$  based on a specific activity of 103  $\mu\text{Ci./mg.}$ ). While this amount of HMVA (1.0 mg.) should have caused considerable swamping, the actual effect was a 15% decrease in mevalonate conversion (tube 9 and 10) as compared to that observed when labeled MVA was incubated in the absence of HMVA (tube 6).

An attempt to swamp the incorporation of HMVA by the addition of 1.0 mg. unlabeled MVA was successful in that there was an 85% decrease in the conversion of labeled substrate (tubes 11 and 12) as compared to that observed in a similar incubation done in the absence of mevalonate (tubes 2 and 3).

#### Experiment 2 - Effect of Buffer on Homomevalonate Incorporation

The pH and composition of the buffer used in the incubation medium had a marked effect on the ability of the enzyme system to catalyze the incorporation of homo-

mevalonate.

The optimum pH for HMVA utilization, as shown in Figure 9, was 7.2 when Tris-HCl buffer was employed. A change to the use of phosphate buffer (pH 7.5) resulted in a 4-fold reduction in the incorporation of homomevalonic acid as compared to Tris-HCl buffer at the same pH.

The levels of incorporation for the ether extracts and the farnesol bands are shown in Table 10. Mevalonate controls indicated that the enzyme preparation was active, while the blanks showed that precursor was not extracted.

Figure 10 shows a scan of the radioactivity found on the chromatogram of a sample resulting from the incubation of HMVA with Tris-HCl buffer, pH 7.4. Band A is nerolidol; bands B are farnesol; band C is geraniol. Peak 1 is the band designated as "homofarnesol." Peak 2, which may be an intermediate, varied in amount depending upon conditions.

### Experiment 3 - ATP Parameter

The amount of ATP present in the incubation medium played an important role in the conversion of HMVA to radioactive product. As the number of  $\mu$ moles of ATP ranged from 7 to 21, the percent incorporation of HMVA into petroleum ether-soluble compounds rose from a level of 2% to the optimum, at 21  $\mu$ moles, of 5%. When ATP was added at a level greater than 21  $\mu$ moles, it had an inhibitory effect as shown in Figure 11.

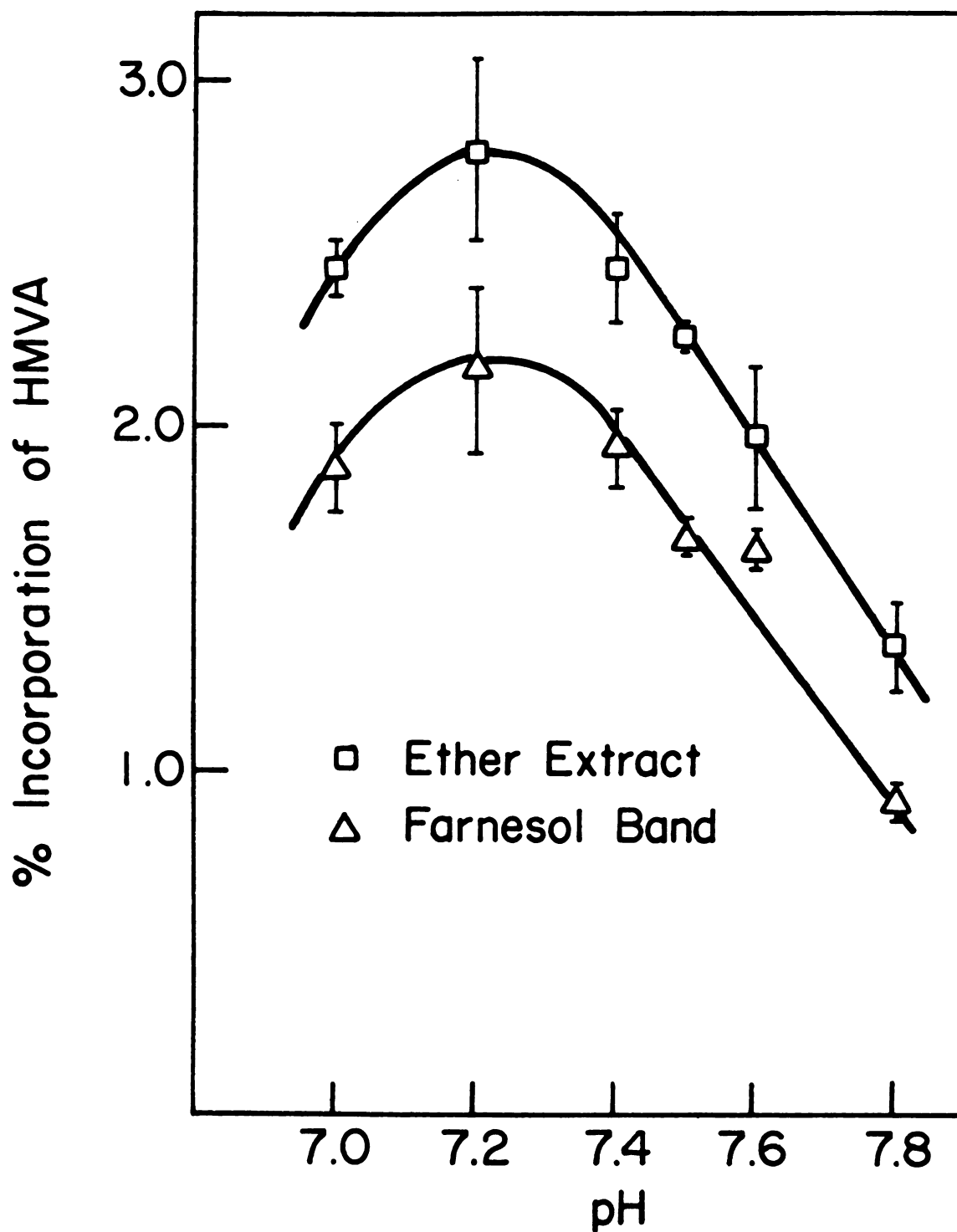


Figure 9. pH parameter for HMVA incorporation. The ether curve represents ether extractable counts while the farnesol curve shows counts in the homofarnesol band.

Table 10  
Homomevalonate Incorporation: pH Parameter

| Sample   | Buffer    | pH  | % Incorporation <sup>a</sup> |      |                            |      |
|----------|-----------|-----|------------------------------|------|----------------------------|------|
|          |           |     | Ether Extract                |      | Farnesol Band <sup>b</sup> |      |
|          |           |     | Avg                          |      | Avg                        |      |
| H*       | Tris-HCl  | 7.0 | 2.38                         |      | 1.75                       |      |
| H*       |           | 7.0 | 2.54                         | 2.46 | 2.01                       | 1.88 |
| H*       |           | 7.2 | 2.54                         |      | 1.92                       |      |
| H*       |           | 7.2 | 3.07                         | 2.80 | 2.40                       | 2.16 |
| H*       |           | 7.4 | 2.30                         |      | 2.05                       |      |
| H*       |           | 7.4 | 2.61                         | 2.45 | 1.83                       | 1.94 |
| H*       |           | 7.5 | 2.24                         |      | 1.64                       |      |
| H*       |           | 7.5 | 2.27                         | 2.26 | 1.71                       | 1.68 |
| H*       | Phosphate | 7.6 | 1.76                         |      | 1.68                       |      |
| H*       |           | 7.6 | 2.18                         | 1.97 | 1.58                       | 1.63 |
| H*       |           | 7.8 | 1.49                         |      | 0.86                       |      |
| H*       |           | 7.8 | 1.24                         | 1.37 | 0.95                       | 0.91 |
| H*       | Tris-HCl  | 7.5 | 0.52                         |      | 0.32                       |      |
| H*       |           | 7.5 | 0.50                         | 0.51 | 0.38                       | 0.35 |
| H* blank | Tris-HCl  | 7.5 | 0.20                         |      | ---                        |      |
| M* blank |           | 7.5 | 0.60                         |      | ---                        |      |
| M*       |           | 7.5 | 42.4                         |      | 37.3                       |      |
| M*       |           | 7.5 | 52.3                         | 47.3 | 43.2                       | 40.3 |

<sup>a</sup>Based on 50% of the radioactivity added: 540,000 dpm mevalonate (M\*) and 912,000 dpm homomevalonate (H\*).

<sup>b</sup>This band is "homofarnesol" in the tubes from homomevalonate incubations.

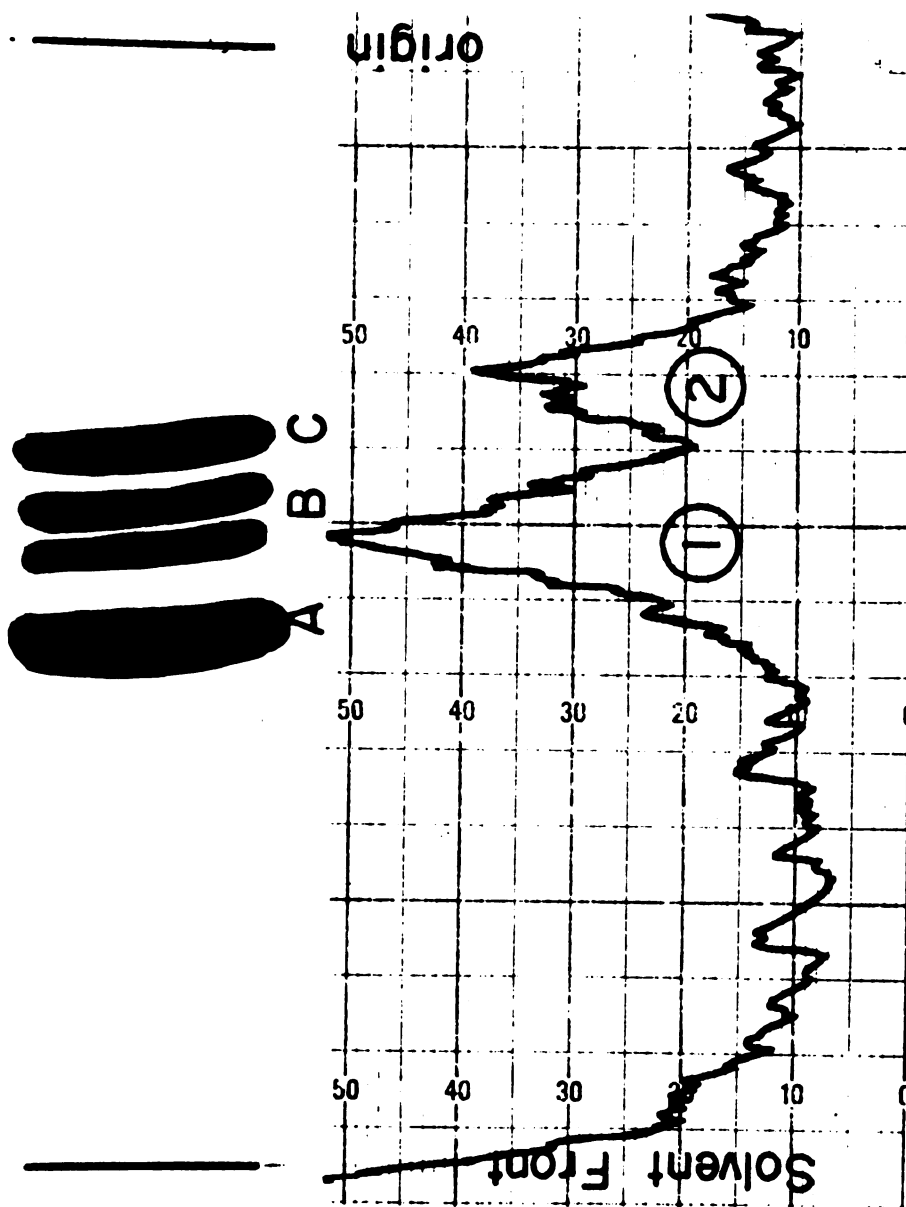


Figure 10. Strip scan for radioactivity on a thin-layer chromatogram from HMVA incubation. A is nerolidol; B is farnesol; C is geraniol. Peak 1 is homofarnesol while peak 2 may be another intermediate. Solvent was benzene/ethyl acetate (4:1).

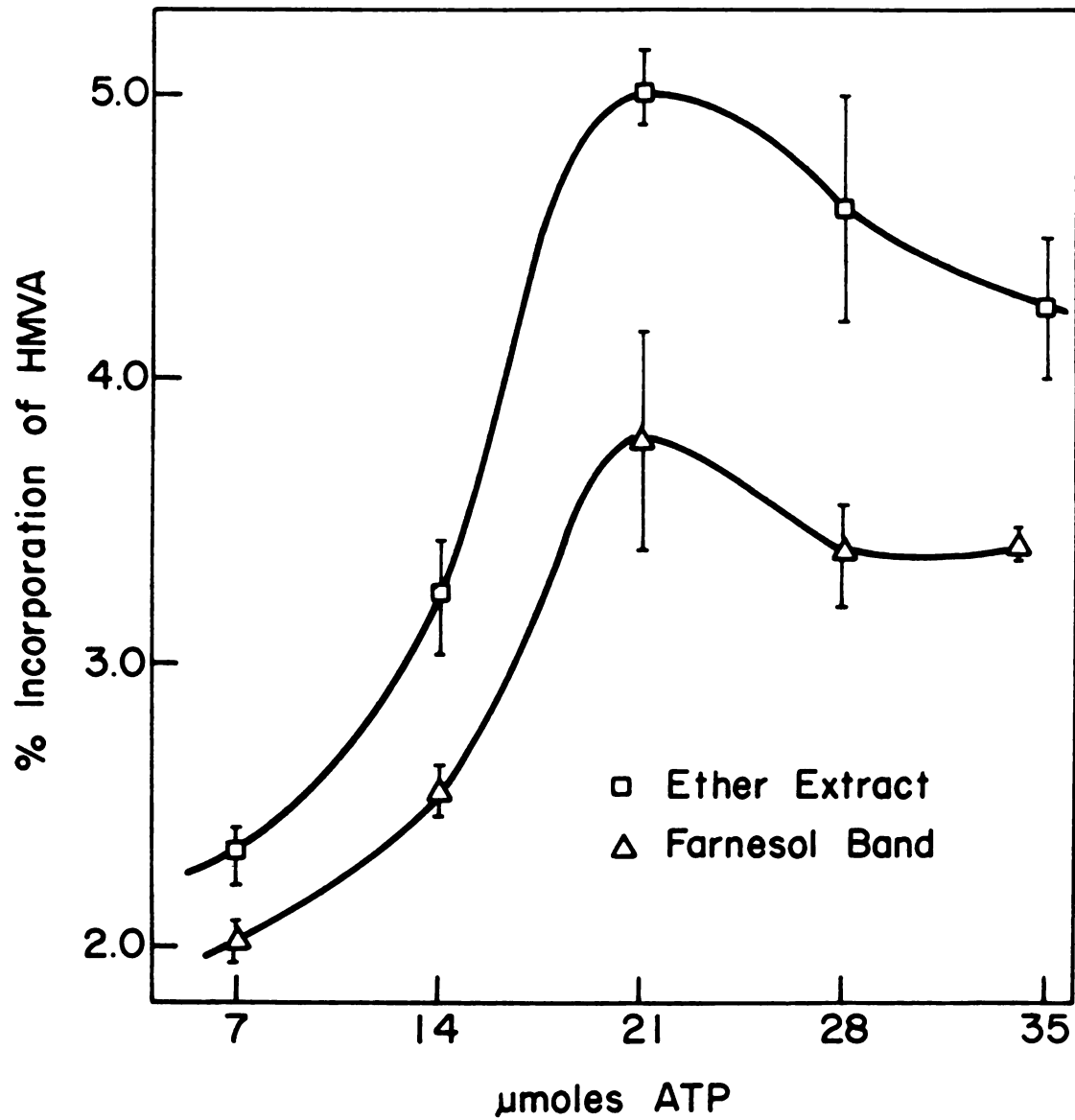


Figure 11. ATP parameter for HMVA incorporation. The ether curve represents ether extractable radioactivity while the farnesol curve shows counts in homofarnesol.

The addition of sodium fluoride resulted in a greater recovery of radioactivity in the petroleum ether extract. This is demonstrated by the fact that samples incubated with 21  $\mu$ moles ATP and pH 7.2 buffer in the previous experiment showed a 2.8% conversion of substrate into petroleum ether-soluble compounds, whereas the same combination in this experiment yielded a 5% conversion. Furthermore, the NaF might have caused the disappearance of the lower radioactive band, since it was not seen in this experiment except at very high levels of ATP.

Table 11 shows the distribution of radioactivity in the samples studied. The blanks showed low incorporation indicating that the precursor was not extracted. Enzyme activity was adequately demonstrated by the incubations with mevalonate.

#### Experiment 4 - Effect of Magnesium Chloride

Increasing the amount of magnesium chloride from 5  $\mu$ moles to 20  $\mu$ moles resulted in a steady increase in HMVA incorporation. An optimum was not reached although HMVA utilization seemed to be leveling off at the 20  $\mu$ mole incubation (Figure 12). Table 12 shows minimal activity in the blanks and good enzyme activity in the mevalonate conversions.

To further elucidate the properties of the labeled substance formed from HMVA, an aliquot of the sample showing optimal conversion of homomevalonate was chromatographed

Table 11  
ATP Parameter for HMVA Incorporation

| Substrate | $\mu$ moles ATP | % Incorporation <sup>a</sup> |      |               |      |
|-----------|-----------------|------------------------------|------|---------------|------|
|           |                 | Pet Ether Extract            |      | Farnesol Band |      |
|           |                 | Avg                          |      | Avg           |      |
| H*        | 7               | 2.21                         |      | 1.94          |      |
| H*        | 7               | 2.42                         | 2.32 | 2.09          | 2.02 |
| H*        | 14              | 3.03                         |      | 2.63          |      |
| H*        | 14              | 3.43                         | 3.23 | 2.46          | 2.55 |
| H*        | 21              | 5.16                         |      | 4.17          |      |
| H*        | 21              | 4.90                         | 5.03 | 3.40          | 3.78 |
| H*        | 28              | 4.21                         |      | 3.20          |      |
| H*        | 28              | 5.04                         | 4.62 | 3.56          | 3.33 |
| H*        | 35              | 4.5                          |      | 3.43          |      |
| H*        | 35              | 4.0                          | 4.25 | 3.47          | 3.45 |
| M*        | 21              | 74.1                         |      | 86.5          |      |
| M*        | 21              | 92.9                         | 83.5 | 94.1          | 90.3 |
| M* blank  | 21              | .32                          |      | .05           |      |
| H* blank  | 21              | .45                          |      | .23           |      |

<sup>a</sup>Based on 50% of the radioactivity added: 1,400,000 dpm homomevalonate (H\*) and 1,190,000 dpm mevalonate (M\*).

<sup>b</sup>This band is "homofarnesol" when homomevalonate is substrate.

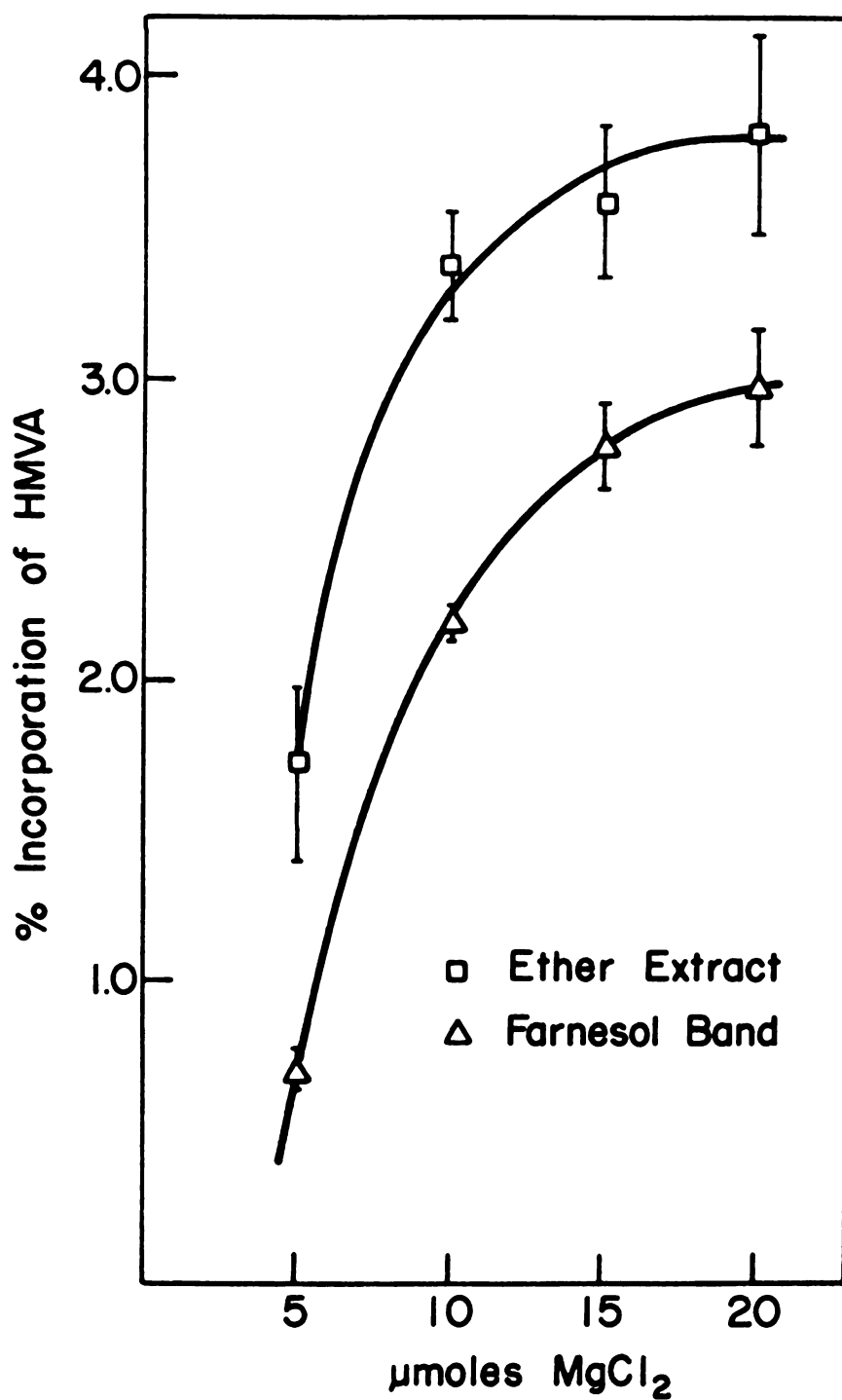


Figure 12. Effect of magnesium chloride on HMVA utilization. The ether curve represents ether extractable radioactivity while the farnesol curve shows counts in homofarnesol.

Table 12

## Magnesium Chloride Enhancement of HMVA Incorporation

| Sample   | $\mu$ moles $MgCl_2$ | % Incorporation <sup>a</sup> |      |                            |      |
|----------|----------------------|------------------------------|------|----------------------------|------|
|          |                      | Pet Ether Extract            |      | Farnesol Band <sup>b</sup> |      |
|          |                      | Avg                          |      | Avg                        |      |
| H*       | 5                    | 1.98                         |      | 0.78                       |      |
| H*       | 5                    | 1.40                         | 1.69 | 0.64                       | 0.72 |
| H*       | 10                   | 3.56                         |      | 2.25                       |      |
| H*       | 10                   | 3.20                         | 3.38 | 2.12                       | 2.19 |
| H*       | 15                   | 3.33                         |      | 2.64                       |      |
| H*       | 15                   | 3.84                         | 3.59 | 2.93                       | 2.79 |
| H*       | 20                   | 4.14                         |      | 3.17                       |      |
| H*       | 20                   | 3.49                         | 3.82 | 2.78                       | 2.97 |
| H* blank | 10                   | .31                          |      | ---                        |      |
| M* blank | 10                   | .001                         |      | ---                        |      |
| M*       | 10                   | 72.5                         |      | 44.5                       |      |
| M*       | 10                   | 59.8                         | 66.0 | 36.8                       | 40.6 |

<sup>a</sup>Based on 50% of the radioactivity added: 1,400,000 dpm homomevalonate (H\*) and 1,190,000 dpm mevalonate (M\*).

<sup>b</sup>This band is "homofarnesol" when homomevalonate is the substrate.

in benzene/ethyl acetate. The band of silica gel adjacent to the farnesol marker was scraped into a Pasteur pipette fitted with a glass wool plug. The labeled compound was eluted with diethyl ether and the eluate divided into two aliquots: one aliquot was rechromatographed in benzene/ethyl acetate, and the other in hexane/diethyl ether. In both of these systems, the radioactivity was observed at an  $R_f$  slightly higher than that of farnesol. The amount of radioactivity recovered from each of these bands was the same within the limits of experimental error.

#### G. Effect of Terpene Volatility on Recovery of Radioactivity

##### Experiment 1 - Scintillation Counting

Aliquots of benzene solutions of labeled prenols were routinely assayed for radioactivity by scintillation counting. Since benzene is a quenching agent, it had to be removed from samples before scintillation fluid could be added. Therefore, aliquots of the benzene solutions were blown to dryness in scintillation vials under a stream of nitrogen. The large surface area of the vial, combined with the small amounts of the labeled compounds present, could result in loss of counts due to the evaporation of volatile prenol. Therefore, all samples were made up in toluene which could be added directly to scintillation fluid. That the latter procedure resulted in greater recovery of radioactivity is shown in Figure 13.

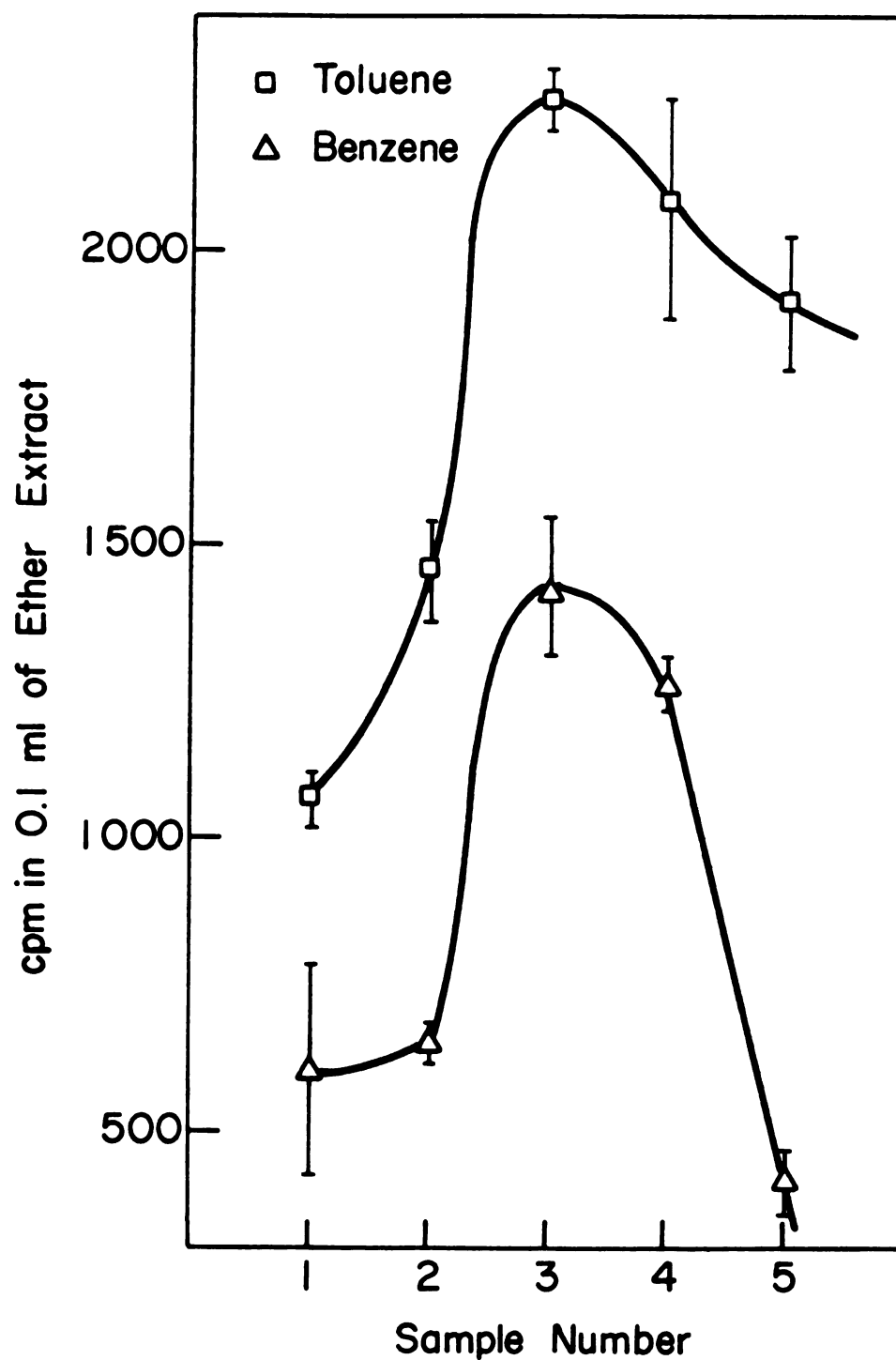


Figure 13. Effect of evaporation on scintillation counting. The lower curve resulted from benzene samples which were evaporated before the addition of scintillator while the upper curve shows toluene samples added directly to scintillator.

## Experiment 2 - Thin-Layer Chromatography

Another procedure that was affected by the volatility of prenols was thin-layer chromatography. Figure 14 shows two scans of a chromatogram of prenols: the lower scan was taken immediately after the completion of chromatography; the other, after the chromatogram had been allowed to stand at room temperature for 24 hours. Up to 60% of the radioactivity was lost during this time period.

### H. Effect of Acid Treatment on Prenols

This study was carried out to determine whether the use of acid to liberate prenols resulted in the appearance of a radioactive band that was less polar than nerolidol. A band with these properties was consistently observed whenever acid treatment was employed. Figure 15 shows that acid treatment of labeled farnesol had the expected effect.

### I. Conversion of Prenyl Pyrophosphates to Sterol

This experiment was designed to determine whether HMVA formed a prenyl pyrophosphate that could be converted to a squalene or sterol analog. Since farnesyl pyrophosphate and the analog prepared from HMVA were not commercially available, these compounds were biosynthesized in incubations which had MVA or HMVA added to the media. The pyrophosphate mixtures were isolated by the method of Rilling and Sofer (19) and added to a squalene synthesizing system

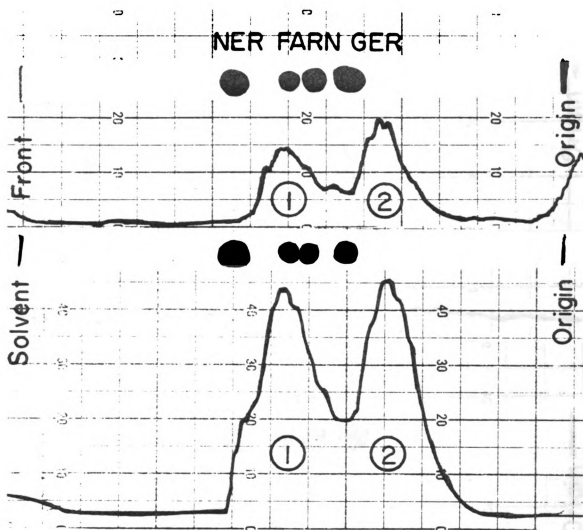


Figure 14. Effect of evaporation on scans for radioactivity. The lower scan was taken immediately after chromatography; the upper scan of the same plate was taken 24 hours later. Peaks 1 and 2 were homofarnesol and intermediate. Markers were nerolidol (NER), farnesol (FARN), geraniol (GER). Solvent was benzene/ethyl acetate (4:1).

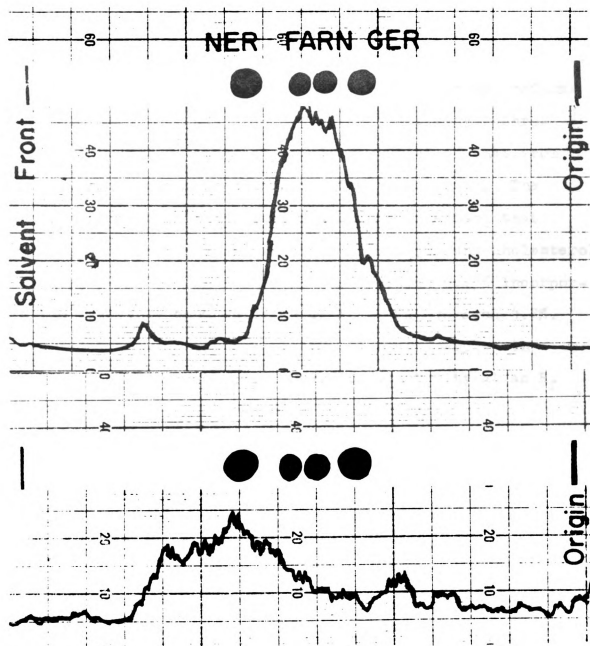


Figure 15. Effect of acid on labeled farnesol. Radioactive farnesol was chromatographed in benzene/ethyl acetate (4:1) as shown in the upper scan. After acid treatment, the labeled products resulted in the lower scan. Markers were nerolidol (NER), farnesol (FARN), geraniol (GER).

prepared from rat liver. Petroleum ether extracts of the squalene incubation media had an average incorporation of 0.41% of the HMVA precursor and 12.8% of the MVA precursor. Thin-layer chromatography of these extracts in chloroform/acetone revealed that some of the radioactivity cochromatographed with the added squalene: 0.04% of that from homomevalonate and 1.53% of that from mevalonate. The remainder of the radioactivity appeared in a band that had an  $R_f$  midway between those of lanosterol and cholesterol. The extracts from HMVA incubations averaged 0.15% incorporation into this band while those from MVA averaged 4.2%. Chromatography of another aliquot of these samples in hexane/diethyl ether again showed radioactivity at an  $R_f$  corresponding to that of the sterol markers.

## DISCUSSION

Since it was not known whether homomevalonate would be acted upon by the specific enzymes that catalyze the conversion of mevalonate to cholesterol, the initial experiments recorded in this thesis were carried out with crude enzyme preparations (from yeast and animal sources) that were previously reported to incorporate mevalonate.

The first yeast enzyme experiment was an in vitro study designed to test MVA uptake and resulted in low overall incorporation of labeled substrate into ether-soluble terpenes. This could be explained if prenyl pyrophosphates were formed, since these water-soluble compounds were not hydrolyzed by saponification with ethanolic KOH (15).

Proof that pyrophosphates were formed was obtained from the second yeast experiment which showed that alkaline phosphatase treatment of samples incubated with MVA resulted in a 10-fold increase in the ether extractable radioactivity. The majority of the counts in the ether extract were eluted from an alumina column into the sterol fraction. When 2-<sup>14</sup>C-homomevalonolactone was incubated in this system, all the ether-soluble radioactivity was eluted into the precursor fraction. Thus, homomevalono-

lactone was inactive as a substrate for the yeast enzyme preparation--an observation that is not surprising since Popjak reported that mevalonolactone was inactive in enzyme systems (37).

In the third yeast experiment, free homomevalonic acid was added as a substrate in an attempt to assess its activity under more valid conditions. No conclusion could be drawn since little activity was observed with either MVA or HMVA. Low radioactivity in all of the terpene fractions could be explained by a combination of the following observations:

1. Squalene is optimally synthesized under anaerobic conditions (38). Incubations in this experiment were aerobic; therefore, squalene was not expected to accumulate.
2. The samples in this series were not saponified; therefore, any labeled sterol might be trapped as the ester which would migrate at an  $R_f$  different from that of other terpenes. This possibility was not checked since only bands corresponding to free cholesterol, squalene and prenol were counted. However, large amounts of sterol ester were not expected.
3. Later studies, reported in this thesis, showed that the complete evaporation of solvent from ether extracts of labeled prenols resulted in loss of radioactivity. Thus, the prenols in this experiment could have been lost when the ether extracts were taken to dryness on a rotary evaporator.

The last observation is the most likely explanation for the lack of incorporation in this experiment since prenols were the first radioactive products formed.

GLC- $^{14}\text{C}$  analysis was used to evaluate the results of

the fourth yeast experiment and demonstrated the incorporation of radioactivity into farnesol, nerolidol, and squalene - thus confirming most of the results of previous TLC studies. However, previous TLC studies of neutral lipid fractions had routinely shown the presence of a radioactive band assumed to be cholesterol. Closer examination of this band revealed that it might be farnesol liberated from farnesyl pyrophosphate by the action of phosphatase. This is plausible since microsomes were not removed from the enzyme preparation, and a phosphatase active with farnesyl pyrophosphate has been found by Goodman and Popják (15). Liberated farnesol could also account for the large amount of radioactivity observed in the solvent peak during GLC- $^{14}\text{C}$  analysis of the neutral lipid samples.

The final yeast experiment was an unsuccessful attempt to assess the value of HMVA as an inhibitor of MVA by spectrophotometric methods. However, the failure of kinetic studies employing crude homogenates is well documented by the work of Popják et al. (40). They attributed their failure to the presence, in the crude homogenates, of glycolytic enzymes which acted on the glycogen from microsomes to form glucose-6-phosphate. This in turn, was a substrate for glucose-6-phosphate dehydrogenase action which resulted in the formation of NADPH. Thus, the absorbance reading of NADPH at 340 nm.

would remain constant or decrease at a constant rate in all tubes containing the same incubation media. This explains the results of the yeast experiment which was followed at 340 nm.

The yeast experiments did not allow any conclusions to be made concerning the biological activity of HMVA; therefore, two experiments with rat liver enzymes were performed. The first experiment, in which acid was used to liberate prenol from pyrophosphate, resulted in the extraction of a large amount of a labeled compound which behaved like homomevalonolactone or mevalonolactone on a thin-layer chromatogram. Thus, acid treatment (low pH) resulted in the cyclization of mevalonic acid and homomevalonic acid to their respective lactones. This behavior is verified by Gray's use of acid-catalyzed lactonization in the initial synthesis of labeled homomevalonolactone (39).

The second experiment using rat liver enzymes was a test of HMVA as an inhibitor of MVA utilization. At least 90% inhibition was expected from the presence of HMVA in 100-fold excess of MVA; however, only 20% inhibition was observed. The inhibition was attributed to the phosphate in the buffer used to dissolve HMVA, especially since Popják's laboratory has documented phosphate as an inhibitor of MVA utilization (15). Thus HMVA would not appear to inhibit MVA incorporation. This observation, if

true, would agree with the report by Takai and Tamura that homomevalonate did not inhibit the growth of Lactobacillus acidophilus when mevalonate was added to the media (32).

The difficulties encountered in the use of crude enzyme preparations from rat liver and yeast led to studies with a soluble enzyme fraction from pig liver. The stability of this fraction permitted the use of a single enzyme preparation in all experiments subsequently discussed in this thesis.

The first experiment with pig liver demonstrated incorporation of HMVA into a petroleum ether-soluble fraction which gave two bands in TLC analysis; one (postulated to be "homofarnesol") which had an  $R_f$  slightly higher than that of farnesol, and the other (postulated to be an intermediate to homofarnesol) which had an  $R_f$  slightly lower than that of geraniol.

The formation of these bands had a pH optimum at 7.2 in Tris-HCl buffer, an ATP optimum of 21  $\mu$ moles, and a stimulation by magnesium chloride that seemed to level at 20  $\mu$ moles. The type of buffer was very critical for the conversion of HMVA into homofarnesol since the use of phosphate buffer resulted in a 4-fold decrease of radioactive product. Since the pH optimum for mevalonate incorporation is 7.5 (15) and that for incorporation of the analog is 7.2, a lower pH might be needed to cause a favorable change in the conformation of the enzyme to allow accomodation of

the ethyl group of homomevalonate.

That ATP is needed for homofarnesol synthesis is shown by the fact that radioactivity in this band was minimal when low concentrations of this cofactor were employed. However, higher concentrations of ATP above the optimal level inhibited homofarnesol synthesis and seemed to result in the appearance of more radioactivity at a lower  $R_f$ . Thus, ATP at high concentrations might exert feedback inhibition on some stage of the biosynthesis of homofarnesol and result in accumulation of a labeled intermediate.

Magnesium was required for homofarnesol synthesis just as for farnesol synthesis. Low concentrations of this ion resulted in low incorporation into the homofarnesol band. An optimum for this ion has not yet been found but the stimulatory effect seemed to level at 20  $\mu$ moles.

That homomevalonate was not the preferred substrate for the pig liver enzyme system was shown by the fact that a 400-fold excess of HMVA caused only a 15% decrease in the utilization of 2- $^{14}$ C-MVA, whereas the same excess of MVA caused an 84% decrease in the uptake of 2- $^{14}$ C-HMVA.

Factors leading to the postulation of homofarnesol synthesis from homomevalonate were the following:

1. Petroleum ether extracts of HMVA incubation media yielded a compound which chromatographed slightly higher than marker farnesol in benzene/ethyl acetate. This band, when rechromatographed, yielded a compound which chromatographed slightly higher than marker farnesol in benzene/ethyl acetate.

tographed in benzene/ethyl acetate and hexane/diethyl ether, again showed an  $R_f$  slightly higher than farnesol did.

2. This compound required magnesium ions and ATP for synthesis.
3. A preparation of the pyrophosphate of this compound, when incubated in squalene synthesizing system, gave rise to a second compound that chromatographed with sterol in two solvent systems.

That the band at lower  $R_f$  was an intermediate in homofarnesol synthesis was postulated from the following observations:

1. This compound appeared at high concentration of ATP which inhibited homofarnesol synthesis.
2. This compound seemed to disappear when NaF was added to the incubation media to inhibit microsomal phosphatase. The absence of NaF resulted in more of this compound presumably arising from the attack of phosphatase on a pyrophosphate intermediate.

As part of these studies, problems relating to the volatility and acid sensitivity of prenols were resolved. The volatility of prenols made the reproducibility of assays particularly difficult since losses of radioactivity were observed during solvent evaporation, thin-layer chromatography, and scintillation counting. The following techniques were devised to minimize these:

1. Petroleum ether extracts were never taken to dryness on a rotary evaporator but, instead, were concentrated to a volume of 1.0 ml.
2. Petroleum ether concentrates were transferred to one dram vials which were evaporated one at a time under a stream of nitrogen.

3. Prenols were redissolved in toluene which could be added directly to scintillation fluid.
4. All prenol samples were stored in the freezer between analyses.
5. Thin-layer chromatograms of radioactive prenols were not prepared until it was certain that they could be scanned immediately. Radioactive bands were scraped and counted immediately after scanning.

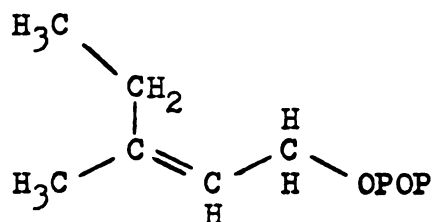
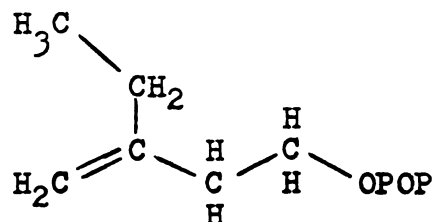
To combat problems caused by acid-catalyzed rearrangement of prenol, Popják's method of acid cleavage of pyrophosphate esters (15) was avoided since it gave several unexplained bands of radioactivity. Instead, alkaline phosphatase treatment was employed since it consistently gave one band of radioactivity from MVA incubations.

The series of experiments with pig liver enzymes demonstrated incorporation of HMVA into a compound postulated to be an analog of farnesol. This compound would arise from a series of intermediates (Table 13) analogous to those involved in cholesterol biosynthesis. If further study does indeed prove the existence of homofarnesol, then another field of study concerning the substrate specificity of terpene biosynthesis has been opened. It is likely that homofarnesol has been synthesized since Popják's laboratory has recently reported that 3-ethyl-3 methyl allyl pyrophosphate was converted to homofarnesyl pyrophosphate by prenyl transferase while 3-propyl-3-methylallyl pyrophosphate was converted to bis-homofarnesyl pyrophosphate (41).

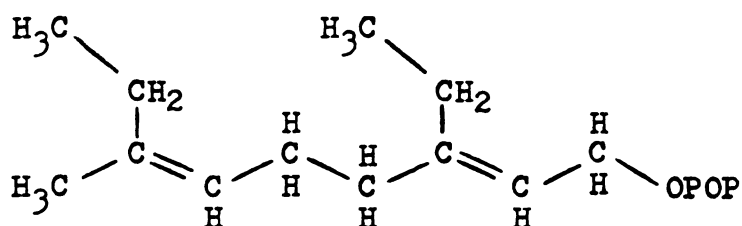
Further work that would be indicated would be a study

Table 13

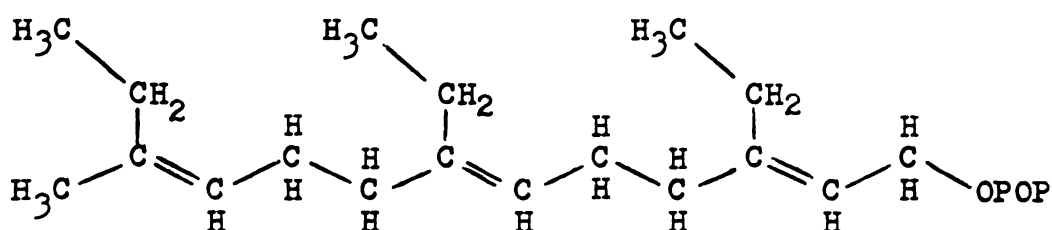
## Intermediates Resulting from HMVA Incorporation

Ethyl methylallyl  
pyrophosphate

Isohexenyl pyrophosphate

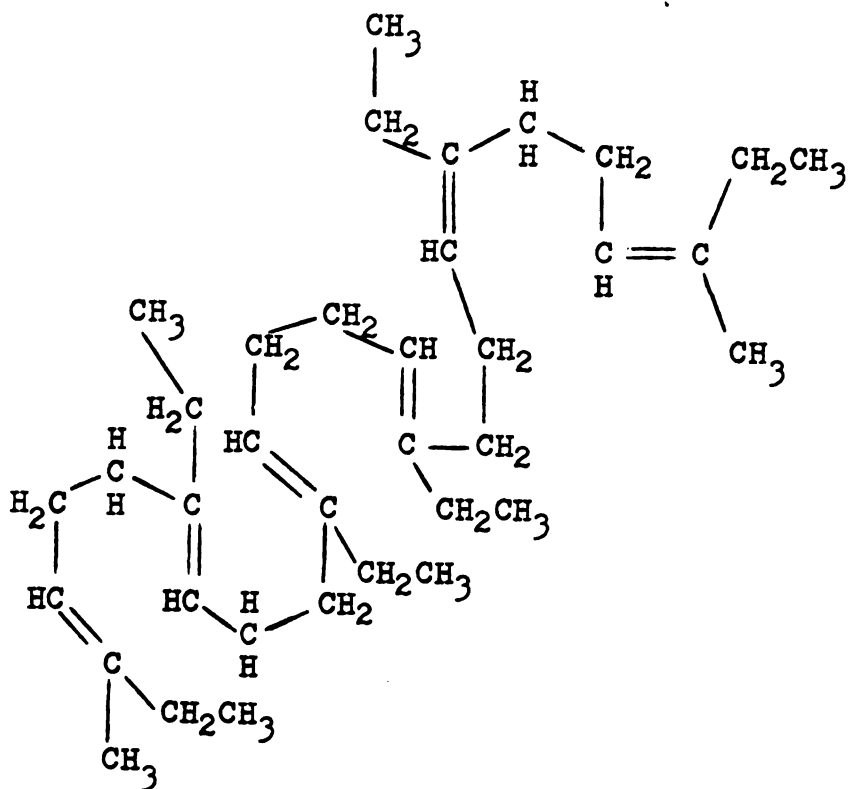


Homogeranyl pyrophosphate



Homofarnesyl pyrophosphate

Table 13 (Continued)



Homosqualene

of derivatives having long hydrocarbon chains at C-3 of mevalonate to determine the maximum length that the enzyme can accept. Furthermore, these additional compounds should be studied over a range of pH to determine if enzyme conformation can be thus altered to accept larger side-chains. If other analogs are accepted as well as homomevalonate, then the presence of these analogs in plant and animal tissues should be researched. Should such analogs be found, then correlations should be sought between amount of analog and possible defects in cholesterol or terpene biosynthesis.

More immediate investigations by GIC-<sup>14</sup>C analysis and mass spectrometry should be done to elucidate the structure of the compound synthesized from homomevalonate. Systems previously studied (rat liver and yeast) should be rechecked for activity with this substrate in the absence of phosphate buffer. Furthermore, investigation should be made of the second labeled compound which is formed at high ATP concentrations or in the absence of NaF.

## SUMMARY

This thesis reports a series of experiments employing homomevalonate as a substrate in enzyme systems known to catalyze the conversion of mevalonate to terpenes--namely the enzymes prepared from yeast, rat liver, and pig liver.

Failure to demonstrate incorporation in the presence of the yeast and rat liver enzymes was subsequently traced to the phosphate buffer employed in the incubation media. Pig liver enzymes prepared in Tris-HCl buffer catalyzed the conversion of HMVA to a compound postulated to be homofarnesol, in a medium which had a pH optimum at 7.2, an ATP requirement of 21  $\mu$ moles, and a stimulation by magnesium chloride at 20  $\mu$ moles. A second labeled compound, appearing at high ATP concentration or in the absence of NaF, is postulated to be an intermediate in homofarnesol biosynthesis.

Mass spectra and gas chromatograms of homomevalonolactone and its TMSi-derivative are reported. The chromatographic behavior of other terpenes on GLC and TLC is also reported.

Finally, techniques are discussed for resolution of problems relating to the volatility and acid-sensitivity of prenols.

STUDIES ON BEESWAX

## INTRODUCTION

Beeswax has been a compound of great biological interest since 1950, when Niemerko and Wlodawer demonstrated that the larvae of the greater wax moth (Galleria mellonella) utilized constituents of beeswax for growth and development (42). Young verified these studies (43) and further demonstrated that the larvae metabolized three of the five homologous series of fatty acids found in wax and excreted the other two series (44).

Young's investigation was aided by the report from Downing's laboratory on the composition of saponified beeswax. These investigators, using the techniques of column and gas chromatography, showed that beeswax contained hydrocarbons, monohydric alcohols, diols, acids, and hydroxy acids (45). The hydrocarbon fraction was shown by mass spectrometry to contain n-paraffins ( $C_{13}$  to  $C_{39}$ ), 2(3)-methyl paraffins ( $C_{14}$ - $C_{38}$ ), 11-methyl paraffins ( $C_{15}$  to  $C_{38}$ ), trans-olefins ( $C_{17}$  to  $C_{31}$ ) and cis-olefins ( $C_{15}$  to  $C_{39}$ ) (46).

Studies on beeswax in this thesis were undertaken primarily to elucidate the structure of the diol fraction reported by Downey. However, the metabolism of beeswax by wax moth larvae prompted a search for terpenes in beeswax

as a key in determining whether Galleria synthesize prenols or merely ingest them. Furthermore, since these insects are presumed to have juvenile hormone, the wax (being the sole source of nutrition) should contain compounds that could serve as precursor to this hormone. Therefore, the paraffin series was investigated to see if there were any compounds having a carbon skeleton similar to that of the hormone.

## EXPERIMENTAL

### A. Materials

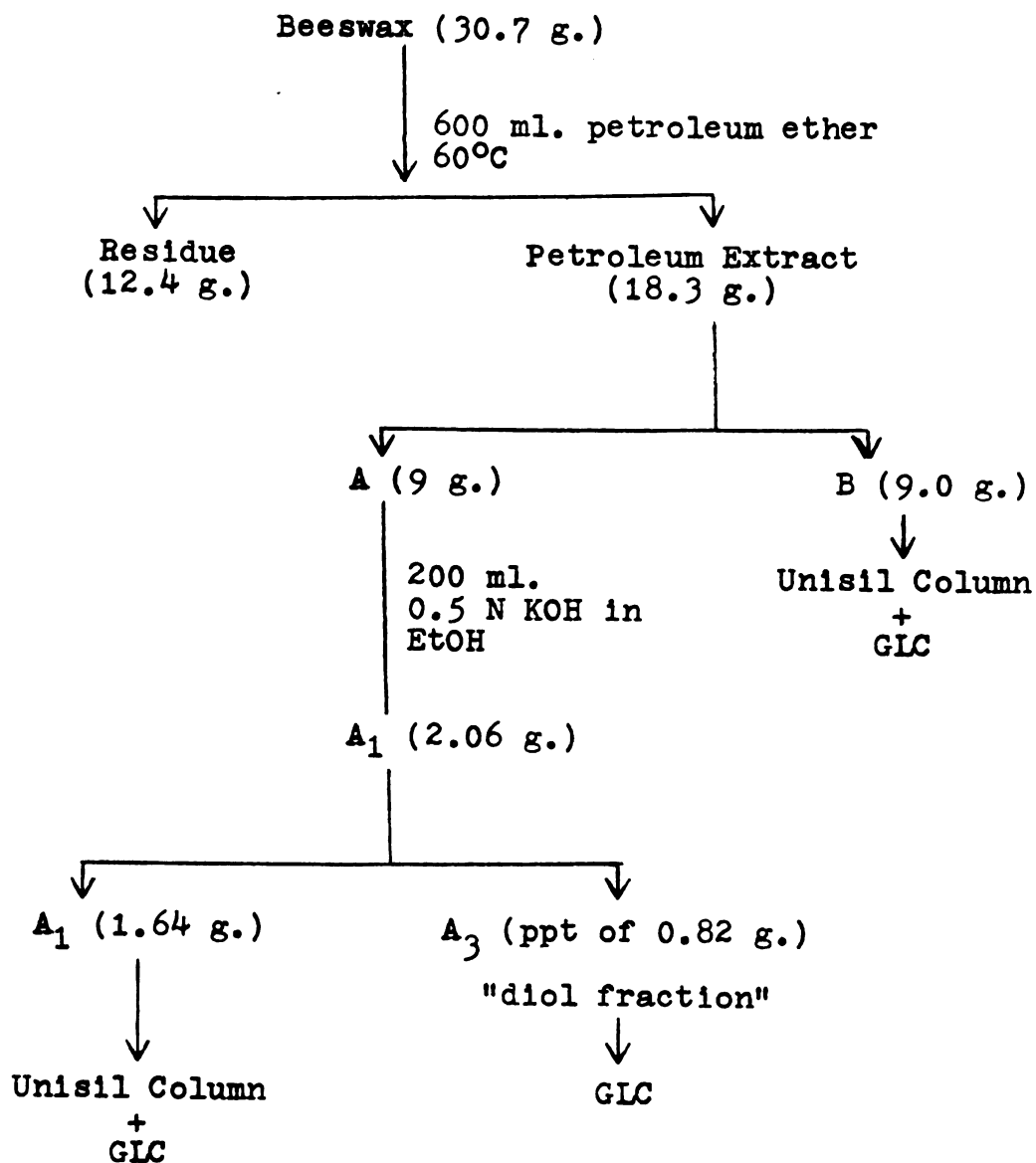
Brood comb was obtained from Mr. Elmer Baumann, Latrobe, Pa. Sources of all other materials were reported in the earlier section of this thesis.

### B. Extraction and GLC Analysis of Brood Comb

Brood comb beeswax (30.7 g.) was extracted with 600 ml. of petroleum ether in a Soxhlet extractor for five hours at 60°C. A grayish-black capsular residue (12.4 g.) remained in the thimble while 18.3 g. were dissolved in the solvent. The wax extract was divided into two 125 ml. portions, A and B, containing 9 g. each. Fraction A was saponified in 200 ml. 0.5 N ethanolic KOH and the saponification medium extracted with petroleum ether to give an unsaponified fraction, A<sub>1</sub>, of 2.06 g. This solution upon standing yielded a white crystalline precipitate of 0.82 g. which was filtered off and analyzed separately as the "diol" fraction. This extraction scheme is shown in Figure 16.

Saponified wax extract (160 mg.) was applied to a 12 g. Unisil column and eluted with 150 ml. of the following solvents; 8% benzene in hexane, 16% benzene in hexane, 25% benzene in hexane, 7% ether in hexane, 50% ether in

Figure 16  
Extraction of Beeswax



hexane, and 2% acetic acid in methanol. Each eluate was evaporated to dryness and weighed. A 10% solution (in the original solvent) was analyzed on 3% OV-1 at 150°C for 10 min. followed by a programmed increase of 2°/minute to 270°C. The weight of each component was determined by comparing its peak area to that of n-eicosane added as an internal standard at a concentration of 1 mg./ml.

Unsaponified wax (700 mg.) was slurried (to avoid pellet formation) into the top of a 12 g. Unisil column. Elution, weight determination, and GLC studies were the same as those of the saponified fraction.

Mass spectra were taken of the hydrocarbon, diol, and 50% ether fractions from saponified wax.

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## RESULTS

### A. Results of Column Chromatography and GLC

#### Experiment 1 - Saponified Wax

Table 14 shows the weight and percent recovery of the fractions from saponified wax. Most of the sample (96%) applied to the column was recovered.

GLC of the 8% benzene fraction from saponified wax (Figure 17) on a 3% OV-1 column programmed from 200° to 270° showed 25 peaks. These were identified by mass spectrometry as even and odd carbon saturated hydrocarbons, even and odd carbon mono-olefins, and two dienes. None of the mass spectra of these compounds showed any resemblance to that of juvenile hormone.

Prenols were sought in the 50% ether fraction since this was the fraction in which farnesol, geraniol, and nerolidol were eluted from Unisil. GLC at 120°C on 3% OV-1 revealed no terpene alcohols in the 50% ether fraction. Programmed GLC (240°-280°) of the TMSi-derivatives of the same fraction revealed peaks which had the same retention times as the major peaks of the TMSi-derivatives of the diol fraction shown in Figure 18 which were chromatographed under the same conditions.

Table 14  
Components of Saponified Beeswax

| Fraction | Solvent                    | Wt. (mg.) | % of Sample Applied <sup>a</sup> |
|----------|----------------------------|-----------|----------------------------------|
| 1        | 8% benzene in hexane       | 41.2      | 26.2                             |
| 2        | 16% benzene in hexane      | 1.9       | 1.2                              |
| 3        | 25% benzene in hexane      | 3.5       | 2.2                              |
| 4        | 7% ether in hexane         | 1.3       | 0.8                              |
| 5        | 50% ether in hexane        | 104.0     | 65.0                             |
| 6        | 2% acetic acid in methanol | 1.6       | 1.0                              |

<sup>a</sup>Based on a weight of 160 mg. applied to the column.

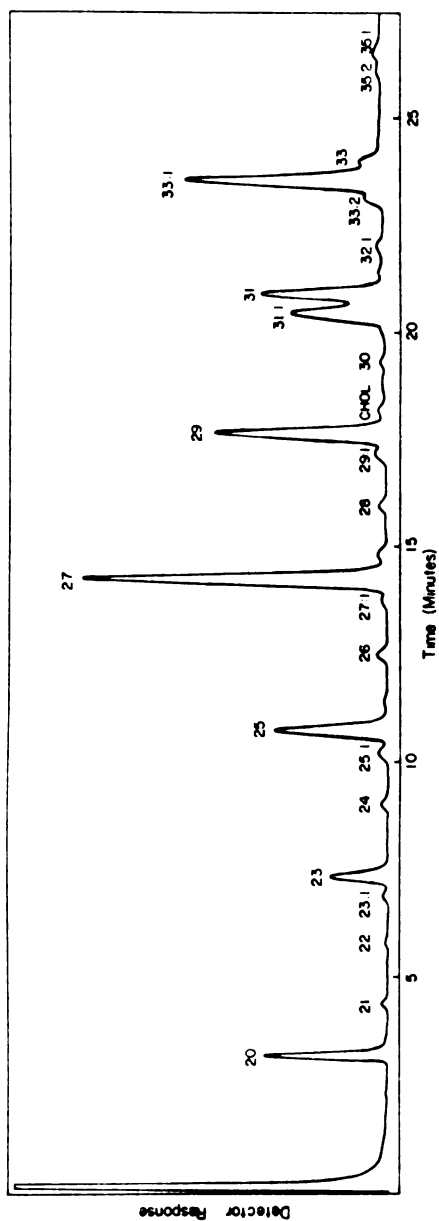


Figure 17. GLC of 8% benzene fraction from saponified wax. [3% OV-1, 200°-270°C].

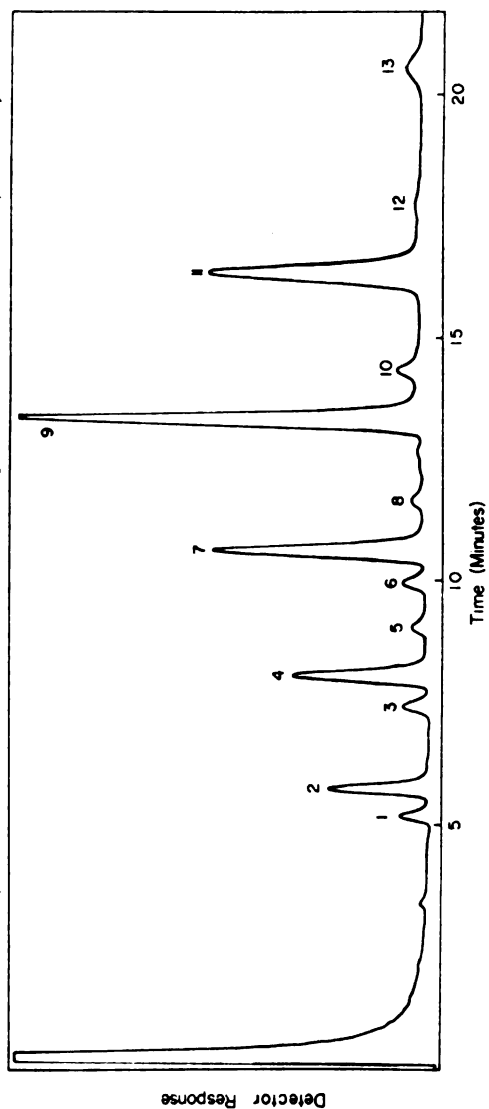


Figure 18. GLC of TMSi-derivatives of the diol fraction from saponified wax. [3% OV-1, 240°-280°C]. Peaks 2, 4, 7, 9, 11, 13 are members of one homologous series. Peaks 5, 8, 10, 12, belong to a second series.

The 16% benzene and 25% benzene fractions were found by GLC to contain hydrocarbons, while the 7% ether and 2% acetic acid fractions showed nothing of interest.

#### Experiment 2 - Unsaponified Wax

Table 15 shows the weight and percent recovery of the fractions from unsaponified wax. Only 60% of the sample applied was recovered from the column. This is probably explained by insufficient elution and column overload.

The fractions of unsaponified wax were analyzed as free compounds and TMSi-derivatives on 3% OV-1 with little success since quantitation showed that most of the sample weight was not eluted from the GLC column. Further analyses were not attempted.

#### B. Mass Spectra of the "Diol Fraction"

This fraction showed two homologous series of compounds: one containing peaks 2, 4, 7, 9, 11, and 13 from Figure 18, and the other, peaks 5, 8, 10, and 12.

Figure 19 is a mass spectrum of peak 7, a member of the first homologous series, and appears to be a spectrum of the TMSi-derivative of a monohydric alcohol. No molecular ion was observed, but a large ion at  $m/e$  467 was assigned to  $[M-15]^+$ , from which a molecular weight of 482 was calculated. This mass is appropriate for the monotrimethylsilyl derivative of a saturated alcohol,  $C_{28}H_{58}O$ .

Table 15

## Components of Unsaponified Beeswax

| Fraction | Solvent                    | Wt. (mg.) | % of Sample Applied <sup>a</sup> |
|----------|----------------------------|-----------|----------------------------------|
| 1        | 8% benzene in hexane       | 286.0     | 41.0                             |
| 2        | 16% benzene in hexane      | 24.4      | 3.1                              |
| 3        | 25% benzene in hexane      | 34.8      | 5.0                              |
| 4        | 7% ether in hexane         | 19.1      | 2.7                              |
| 5        | 50% ether in hexane        | 37.3      | 5.3                              |
| 6        | 2% acetic acid in methanol | 6.9       | 1.0                              |

<sup>a</sup>Based on 700 mg. applied to the column.

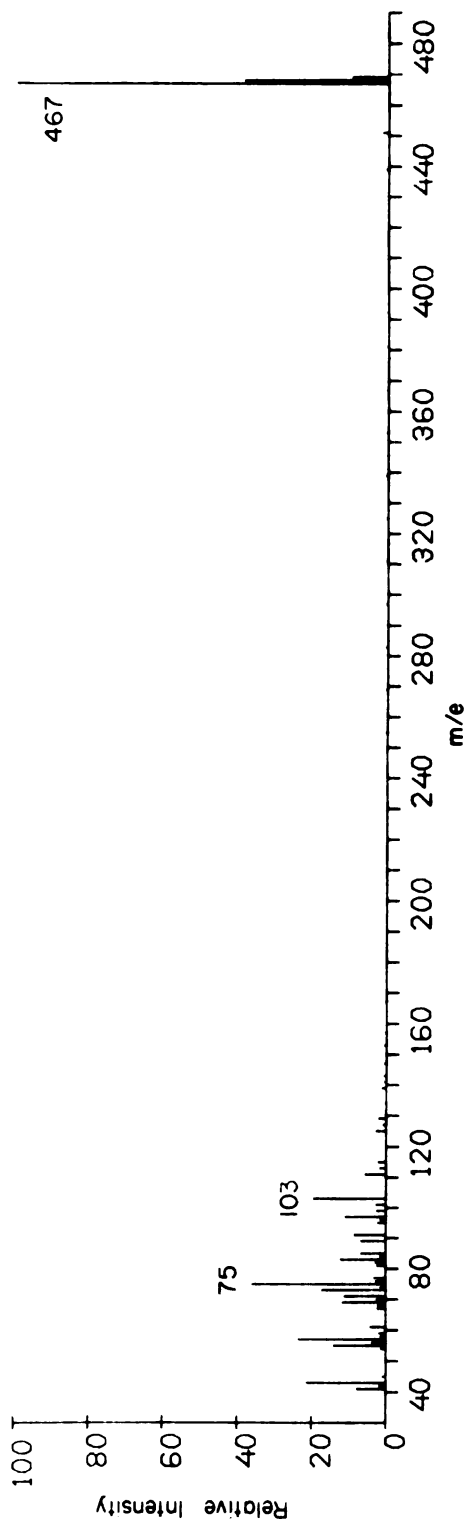


Figure 19. Mass spectrum of TMSi-derivative of diol-7.

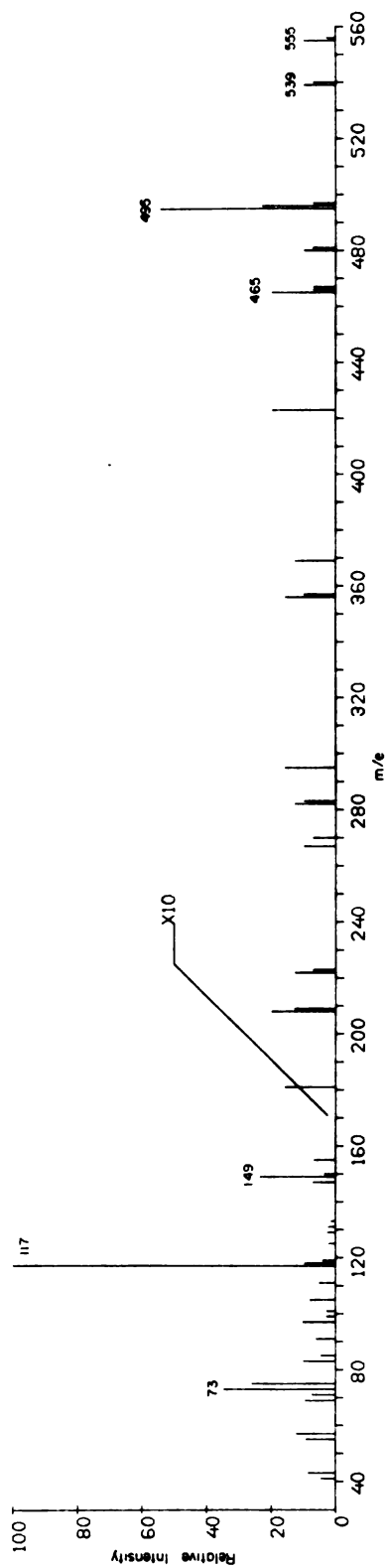


Figure 20. Mass spectrum of TMSi-derivative of diol-10.

There was nothing in the mass spectrum to suggest branching, and the compound was therefore tentatively assigned a normal alkanol structure.

Figure 20 is a mass spectrum of peak 10, a member of the second homologous series and appears to be a spectrum of the TMSi-derivative of a diol. An ion at  $m/e$  555 was assumed to be  $[M-15]^+$ , since other ions at  $m/e$  480  $[M-90]^+$  and  $m/e$  465  $[M-15-90]^+$  were consistent with such a hypothesis. The molecular weight of 570, deduced from these peaks is consistent for the di-trimethylsilyl derivative of a saturated diol,  $C_{28}H_{58}O_2$ , and the very large ion at  $m/e$  117 is strong evidence for the group  $[CH_3CHOTMSi]^+$ . The structure is therefore believed to be a 2,3-diol but other possibilities cannot be excluded. Another compound which could give a di-trimethylsilyl derivative with a molecular weight of 570 is a hydroxy acid,  $C_{27}H_{54}O_3$ , from which a strong ion at  $m/e$  117 might be derived  $[COOTMSi]^+$ . The fact that this homologous series was part of a non-saponifiable fraction makes this an unlikely possibility.

## DISCUSSION

The composition of brood comb found in this study was essentially the same as that reported by Downing; hydrocarbons  $C_{19-35}$ , monohydric alcohols  $C_{24-32}$ , and diols  $C_{24-32}$  (45). Composition of the hydrocarbon fraction agrees with the work of Streibl's laboratory as saturated hydrocarbons  $C_{21-33}$  and olefins  $C_{23-35}$  (46).

The first homologous series in the diol fraction were tentatively assigned normal alkanol structures while the second series were postulated to be saturated diols.

No prenols were found in fractions from beeswax, but the possibility of their occurrence is not ruled out since petroleum ether extraction of beeswax was carried out in an open system by reflux at  $60^{\circ}\text{C}$  - a temperature which might cause loss of volatile prenols. Otherwise, prenols could have been lost when the petroleum ether extracts were taken to dryness on a rotary evaporator - a phenomenon reported in the first section of this thesis.

Evidence for a juvenile hormone-like carbon skeleton was not found in the spectra of the hydrocarbons from beeswax; however, this does not preclude the possibility of such a skeleton appearing in the hydroxy fatty acid fraction.

## SUMMARY

This study of beeswax was undertaken to determine the structure of the diols previously found in beeswax, the existence of prenols in wax, and the existence of a carbon chain similar to that of juvenile hormone in the hydrocarbons of beeswax.

The latter two determinations were negative, while studies on the structures of diols were not conclusive. Compositions of beeswax found in this investigation agreed with the literature reports.

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