# THE ISOLATION OF A $C_{16}$-KETO ACID FROM RAT LIVER DERIVED FROM MEVALONIC ACID

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By

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# A C K N O W L E D G E M E N T

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## ABBREVIATIONS

$CDCl_3$ : Deuterated Chloroform

cpm : Counts per minute

GSH : Reduced form of glutathione

$NAD^+$ : Oxidized form of nicotinamide-adenine dinucleotide

$NADH+H^+$ : Reduced form of nicotinamide-adenine dinucleotide

NSL : Non-saponifiable lipids

# CHAPTER I

## INTRODUCTION

The discovery of mevalonic acid as a remarkably efficient cholesterol precursor in animal tissues (1) and its recognition as a central intermediate in the synthesis of several terpenes and sterols (2) drew the attention to its important metabolic role in animals, yeast, bacteria, and plants.

Although it was assumed that the biosynthetic pathway of mevalonic acid to cholesterol is the major pathway in animals, it became evident after several extensive experiments that mevalonic acid was being incorporated into compounds which do not participate in the cholesterol pathway.

Earliest among these studies recorded was the isolation of an olefinic acid identified as a terpenoid acid (3)enzymatically synthesized by rat liver homogenates from mevalonic acid. Popjak (4) reported the formation of some carboxylic acids namely trans-geraniolic and trans-trans-farnesoic among several other acids following incubation of liver enzyme preparations with mevalonic acid.

Neutral non-carboxylated compounds were isolated as well following incubation with mevalonate in animal tissues. Ubiquinones have been shown by a number of workers to be derived from mevalonate (5), the site of their synthesis in rats being the kidney and

liver specifically (6). The incorporation of mevalonate-2-$^{14}C$ into dolichol in rabbit and pig livers (7) have been investigated and proved as well. More recent studies have indicated the presence of unknown radioactive material (6) presumably a mixture of isoprenoid-like substances following incubation of rat tissue slices with mevalonate-2-$^{14}C$.

Tavrmina and Gibbs(8) proved in studies on mevalonic acid that the radioactivity of mevalonate-1-$^{14}C$ was not incorporated into the non-saponifiable fraction while mevalonate-2-$^{14}C$ yielded radioactive cholesterol. This eventually led to the recognition that decarboxylation occurs at the six carbon atom level to give a five carbon atom active intermediate namely $\Delta^3$-isopentenyl pyrophosphate(9).

Stoichiometric studies on mevalonic acid metabolism in Lactobacillus plantarum (10) showed that for each mole of mevalonate incorporated into the non-saponifiable lipids, one mole of carbon dioxide would be liberated. Studies were also conducted on mammalian adipose tissue (11) and it was found that when various types of adipose tissues were incubated with mevalonic acid-2-$^{14}C$, the results were in agreement with the identified mechanism for the metabolism of mevalonate.The results of these experiments showed that the rate of carbon dioxide paralleled that for the synthesis of non-saponifiable lipids.

The stoichiometry in the metabolism of mevalonate by Lactobacilli could not be demonstrated in adipose tissue.In rat liver, previous studies in this laboratory have shown that for every 3 to 4 moles of labelled carbon dioxide liberated from

mevalonic acid-1-$^{14}C$, 1g-atom of labelled carbon was being incorporated from mevalonic acid-2-$^{14}C$ into the non-saponifiable lipids. So it was assumed that another pathway is operating which involves the incorporation of mevalonate into some unknown substances. The object of this work is to isolate, purify, and identify some of these unknowns.

It became of interest as well to explore the factors which alter the rate of mevalonate incorporation into different substances. Popjak (4) found that upon the addition of $(NAD^{+})_{+}$ $(NADH+H^{+})$ and ascorbic acid to liver enzyme preparation incubated with mevalonic acid, more of the non-saponifiable lipids and carboxylic acids were formed at the expense of polyprenols. Applying such information in this study glutathione (reduced form) , ascorbic acid, cysteine, and phenethylbiguanide were added each separately and with varying concentrations to liver cells incubated with mevalonic acid.

## CHAPTER II

## MATERIALS AND METHODS

### A. Animals

Albino rats of the Sprague-Dawley strain weighing 250-400g were used in these experiments.

### B. Chemicals

All reagents were analytical grade. Radioactive DL-mevalolactone (0.5mC per m mole) was obtained from the Radio Chemical Center, Amersham, England. All other chemicals were obtained from E. Merck, Darmstadt, Germany; Sigma Chemical Co, St. Louis, Mo; and Shandon Scientific Co, Ltd., London.

### C. Preparation of Liver Mince

Rats were anaesthesized and decapitated. Liver tissues were quickly exised from each rat and transferred in pre-cooled beakers containing distilled water to the cold room(temperature 6-8 °C). Liver tissues were quickly minced by a Latapie tissue grinder and aliquots were weighed then placed in incubation flasks. The mince and buffer solutions were kept in the cold room at all times prior to incubation.

### D. Incubation Procedures

1. Factors Affecting the Metabolism of Mevalonate: For the study of the effect of various factors on mevalonate metabolism all incubations were carried out in 50ml Erlenmeyer flasks as described by Bloom et. al.(12) in a Dubnoff shaker at 37 °C for 5 hours with air as the gas phase except where indicated to be different. Duplicates of the same incubation agreed within 5 percent. Each flask contained 3g of liver mince, 4.8 $\mu$ moles of DL-mevalolactone-1-2-$^{14}$C($1.2 \times 10^5$ counts per minute per labelled carbon), 300

$\mu$moles of phosphate buffer pH 7 in a final volume of 6ml. All other conditions are indicated under the appropriate sections. The reaction was terminated by the addition of 0.3ml of 3N sulphuric acid then re-incubated for 15 more minutes.

Assay: The activity of the liver mince was assayed first by the measurement of radioactivity in the carbon dioxide liberated from DL-mevalonate-1-$^{14}C$ as described by Durr and Shwayri(10). This involved diluting the alkali used to trap carbon dioxide and plating 0.1ml of the diluted samples.Concentric planchets were used for counting in a Baird Atomic automatic counter with a sensitivity of $4 \times 10^5$ counts per minute per $\mu$Curie and a background counting rate of 20 counts per minute. The effect of time, pH, mevalonate concentration, anaerobiosis, and the addition of different chemicals were studied.

The second assay involved the extraction of the metabolites derived from mevalonate-2-$^{14}C$ after the addition of reduced glutathione, ascorbic acid, or cysteine in varying concentrations. The contents of each flask -3g of liver mince and 3ml of incubation mixture - were saponified with 6ml of 10% (W/V) KOH in 70% methanol for 2 hours. The mixture was then concentrated to one fifth of its volume, and made up with water to 6ml. 2g of sodium chloride were dissolved, and the resulting saturated solution was extracted with acetone : petroleum ether 7 : 3(V/V). Acetone was then used to extract the metabolites of mevalonate. The acetone extracts were combined and a sample was plated at infinite thinness to determine the radioactivity. The stoichiometry of the carbon dioxide and the metabolites of meva-

lonate will be discussed later.

2. Fractionation of the Metabolites of Mevalonic Acid; For the isolation and purification of the metabolites of mevalonic acid, large scale experiments were run on a total of approximately 1kg of rat liver. 750ml incubation flasks were used each containing 50g of liver mince, 100 $\mu$moles DL-mevalolactone-1-2-$^{14}$C ($3 \times 10^6$ counts per minute per labelled carbon), 3750 $\mu$moles of phosphate buffer pH 7 in a final volume of 200ml. Incubation was carried in a shaker for 5 hours at 37 °C with air as the gas phase in the flasks. The reaction was terminated by the addition of 3ml of concentrated sulphuric acid then re-incubated for 15 more minutes.

Assay: The incubation mixtures were pooled together and centrifuged. The supernate was saved and the wet solid obtained weighed 1300g.

a. Treatment with Acetone: The wet solid was soaked with acetone overnight and filtered under suction. This procedure was repeated three times consecutively. The solid residue, a pale powder, was stored while the filtrate was distilled leaving a dark brown residue. The distillate contained no radioactivity thus discarded.

b. Petroleum Ether Extraction: The dark brown residue was extracted with petrolium ether (B.P.40-60 °C)until no more radioactivity was recovered. The total activity extracted in the petrolium ether layer was approximately $5 \times 10^6$. The aqueous layer was

discarded because of heavy contamination with unreacted mevalonic acid.

c. Saponification: The petroleum ether layer was concentrated down to a thick residue and saponified for 15 hours with 5% (W/V) KOH in 70% methnol. The weight of the saponified fraction was 50g; to it was added 50ml distilled water and concentrated down to half volume to remove all the methanol present.

d. Extraction Under Alkaline Conditions: An additional 50ml of water and 500ml acetone were supplied to the saponified mixture to avoid the formation of emulsions. Several extractions with 2.5 liters of petroleum ether followed until no more radioactivity was obtained in the petroleum ether layer. The total radioactivity in the petroleum ether - acetone layer was $4 \times 10^6$ counts per minute while that of the aqueous-acetone residue was $1 \times 10^6$ counts per minute.

e. Extraction Under Acidic Conditions: To the aqueous-acetone residue 100ml of water were added bringing the final volume to 350 ml. This was acidified to pH 2 by concentrated sulphuric acid, then followed exhaustive extraction with petroleum ether. The residue of this extraction was discarded because of possible contamination with mevalonic acid. The petroleum ether layer was concentrated by flash evaporation to approximately 10ml. The total radioactivity of the concentrated material was approximately $6 \times 10^5$ counts per minute. The radioactive yields of these different fractions are indicated in (Fig.1).

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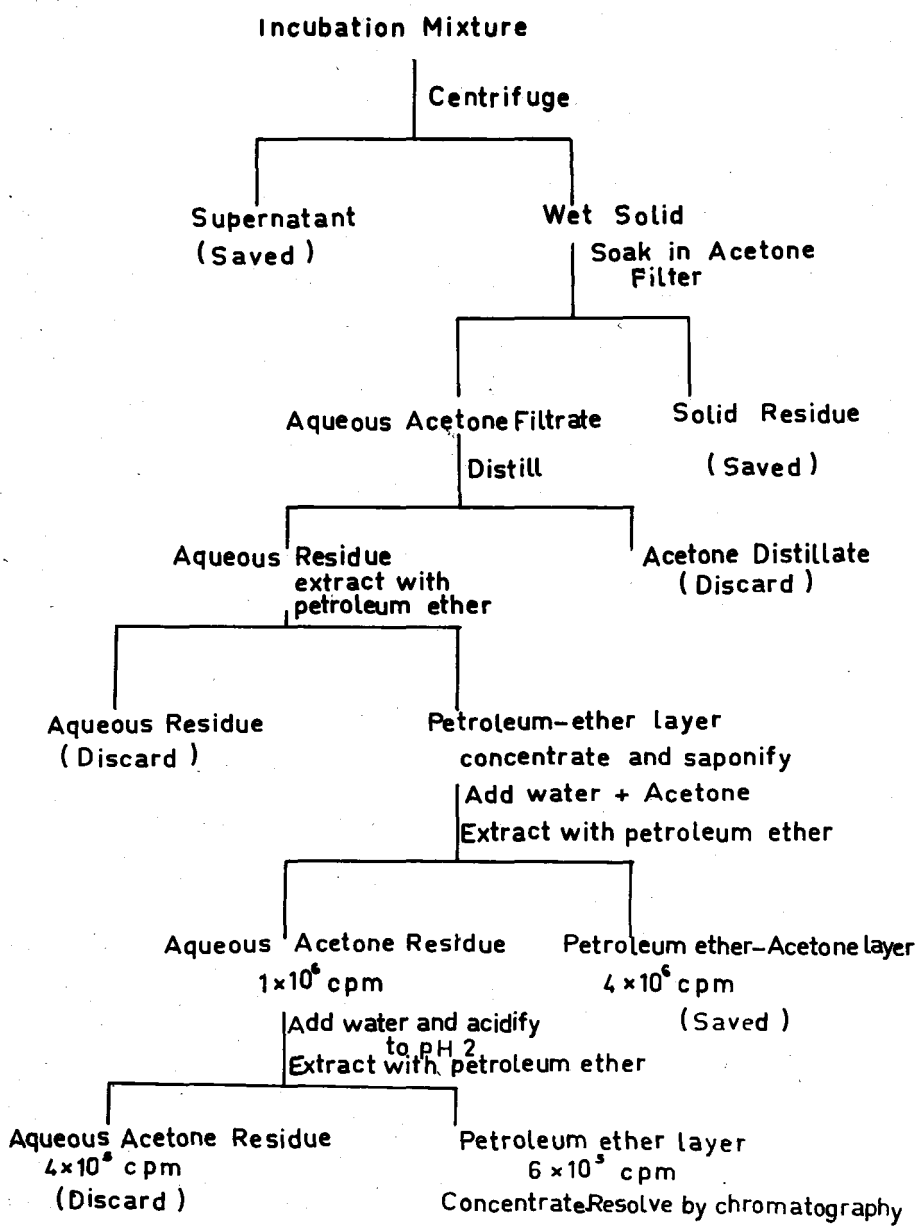



FIGURE I. FLOWSHEET FOR THE ISOLATION OF $X_2$

f. Chromatography: The concentrated petroleum-ether layer was purified by chromatography using:

i. Deactivated Alumina Column: To 100g of standardized aluminum oxide obtained from E-Merck, 7ml of 10% (v/v) acetic acid were added (13). The mixture was suspended in n-heptane and shaken for 6 hours. Filtration followed and the residue was washed with n-neptane 3 to 4 times. The wet powder was left to dry at room temperature. The deactivated alumina was suspended in n-heptane and applied to a column thus registering 3.7x10cm size. The sample was dissolved in 7ml n-heptane and applied to the column. The column was attached to an automatic fraction collector where aliquots, approximately 20ml, were collected every 15 minutes. The column was developed with 100% n-heptane (300ml), 50% (v/v) acetone-heptane (300ml), 100% acetone (300ml), 100% methanol (100ml), 10% (v/v) glacial acetic acid in methanol (600ml), 50% (v/v) glacial acetic acid in methanol (200ml), and finally with 100% glacial acetic acid (400ml). 0.2ml of each sample was plated on stainless steel planchet and counted. The inactive samples were discarded while the radioactive ones were concentrated by flash evaporation and stored.

ii. Silicic Acid Columns: The first fraction collected from the deactivated alumina column, namely the one eluted with 100% n-heptane having total radioactivity of approximately $1x10^5$ counts per minute, was further purified by chromatography on a silicic acid column (14). 100g of silicic acid, obtained

as Bio-Sil-Ha minus 325 mesh from Bio-Rad Laboratories, were suspended in n-heptane. The concentrated fraction was mixed with 5 g of silicic acid and the mixture was applied to 2.5 x 20 cms column. The elution schedule involved at first increasing proportions of diethyl ether in n-heptane and the different fractions were similarly collected by the automatic fraction collector every 25 minutes. First the column was eluted with 100% n-heptane (500 ml) then with 1% (V/V) diethyl ether in n-heptane (500 ml), 5% (V/V) diethyl ether in n-heptane (500 ml), 10% (V/V) diethyl ether in n-heptane (100 ml), 100% diethyl ether (200 ml), 20% (V/V) methanol in diethyl ether (100 ml), 50% (V/V) methanol in diethyl ether (100 ml), and finally with 100% methanol (200 ml). 0.5 ml of each sample was plated on stainless steel planchets and counted. The inactive samples were discarded while the radioactive ones were all pooled together and concentrated down by flash evaporation.

The first two fractions eluted from the silicic acid column namely: 100% n-heptane fraction with total activity of approximately $6.7 \times 10^4$ counts per minute and 1% (V/V) diethyl ether in n-heptane fraction with total activity of approximately $1.1 \times 10^4$ counts per minute were further purified. Attempts to re-crystallize the 100% n-heptane fraction from ethanol-water then diethyl ether failed and the compound decomposed leaving a darkly pigmented oil. Treating half

the 1% diethyl ether in n-heptane fraction similarly did not purify it and the whole fraction was applied to a 10 x 1.5 cm silicic acid column. This column was eluted with 1% (V/V) ethyl acetate in n-heptane (300 ml) and 100% methanol (300 ml). Two main fractions separated out as a result. A white waxy-like crystalline substance, namely $X_1$, which was eluted with 1% ethyl acetate-heptane and a yellow oily substance which was eluted with pure methanol.

iii. Thin Layer Chromatography: To determine the purity and identity of $X_2$ thin layer chromatography was used among several other methods. The silicic acid used for chromatography was Kieselgel G Nach Stahl Cat No. 2860 obtained from Shandon. The silica gel was suspended in distilled water in the ratio 1:2 (by weight) respectively then used for coating the plates (0.20 mm thickness). After standing at room temperature for 15 minutes the plates were transferred to an oven where they were left for 45 minutes at 110°C. Plates were later stored in a dessicator for future use. 0.25$\mu$g of $X_2$ was usually applied to the plates and these were developed by six different solvents namely: 100% n-hexane; ethyl acetate: hexane (3:7 V/V); hexane:ether:glacial acetic acid: methanol (90:20:3:4) (15); hexane:ether:ammonium hydroxide:methanol (90:20:3:4); chloroform:acetone (10:1) and 100% methylene chloride. After drying the plates under an infrared lamp, each was developed in iodine vapor. After all the iodine has

evaporated each plate was sprayed with either 40% (V/V) sulphuric acid or anisaldehyde reagent then developed for 20 minutes at 110 °C.

g. Spectroscopic Studies:

i. Infrared spectra of known substances and various isolated fractions were recorded on a Perkin-Elmer 237 spectrophotometer. They were obtained as thin films of chloroform solutions over NaBr pellets with 0.1 mm cell path.

ii. Ultra-violet spectra were recorded on a Bausch and Lomb Spectronic 505. 95% ethanol was used as solvent.

iii. Nuclear magnetic resonance spectrum of $X_2$ was done by Varian AG, Klausstraße 43, 8008 Zurich-Switzerland. The solvent used was $CDCl_3$.

h. Elementary Chemical Analysis: A microanalysis of $X_2$ dried to constant weight at 50 °C and high vacuum was performed by Dr. Albert Bernhardt Microanalystisches Laboratorium, 433 Mülheim - Germany.

i. Derivative Formation: 2:4-dinitrophenylhydrazine derivative of $X_2$ was prepared as described by Shriner et al (16). The solvent used was 95% ethanol.

j. Esterification: 20 mg of $X_2$ were dissolved in 20 ml 5% (V/V) sulphuric acid in anhydrous methanol and refluxed for 3 hours. The volume was then reduced to 5 ml by flash evaporation and 5 ml of water were added. The mixture was neutralized with saturated $NaHCO_3$ aqueous solution and extracted several times with diethyl ether. The ether layer was then dried over

anhydrous $Na_2SO_4$ and flash-evaporated. Dry nitrogen was applied at room temperature to the residue.

k. Gas-Liquid Chromatography: Esterified X was analyzed by a Pye Argon Chromatograph. The column used was an ethylene glycol-adipate polyester 12.5% on celite 545, 80-100 mesh. The column was straight and 4 feet long. The gas used was argon with a flow rate of 30 ml/minute. The temperature range was 190±192°C.

l. Chemical Tests: Testing for unsaturation in X, bromine water and $KMnO_4$ solution were used according to methods described by Shriner et al (16).

## CHAPTER III

## RESULTS

$CO_2$/NSL Ratio: It was previously mentioned that stoichiometric studies on mevalonic acid metabolism in Lactobacillus plantarum (10) showed that the ratio of $CO_2$/NSL = 1. Further studies were also conducted on mammalian adipose tissue (11) but the $CO_2$/NSL ratio proved to be more than one. It became of interest to determine whether the same is true of liver.

When mevalonate-1 or -2-$^{14}C$ of the same specific activity were incubated with rat or human liver the ratio of $CO_2$/NSL was more than one. This is shown in (table 1).

Since the isolation of certain metabolites of mevalonate was of primary interest, different conditions that may increase their yield were studied. Variations in the incubation period, pH, mevalonate concentration, and different chemical agents were recorded. The activity of the liver mince was assayed by measuring the radioactivity of the $CO_2$ liberated from DL-mevalonate-1-$^{14}C$.

A-Factors Affecting Mevalonate Metabolism

1. Time Study: In order to determine the effect of time on the activity of liver incubation mixture, several flasks each containing 1g of liver mince, 0.25 $\mu$moles of DL-mevalonate-1-2-$^{14}C$ ($1.5 \times 10^5$ counts per minute per labelled carbon), 300 $\mu$moles of phosphate buffer pH 7.5 in a final volume of 3ml were incubated for varying periods of time. The activity of the mince

## Table 1

### $CO_2$/NSL* Ratio As Obtained From Liver Enzyme Systems

| | $C_1$ Counts per minute x $10^{-3}$ | | $C_2$ | | $C_1$ / $C_2$ |
|---|---|---|---|---|---|
| | $CO_2$ | NSL | $CO_2$ | NSL | $CO_2$ / NSL |
| Human Liver | 293 | 0 | 0.96 | 70 | 4.18 |
| Rat Liver | 43 | - | - | 18.80 | 2.30 |

* The $CO_2$ is derived from DL-Mevalonate-1-$^{14}$C while the NSL are derived from DL-Mevalonate-2-$^{14}$C of the same specific activity.

was found to be optimal after 5 hours of incubation (Fig.2). Increasing the incubation period to 6 hours or more yielded a decline in the activity of the liver mince.

2. Effect of pH: Similarly experiments were conducted under various pH values. Similar flasks were incubated for 3 hours and the activity of the liver mince was found to be optimal at pH 6.5 (Fig.3).

3. Effect of Concentration: To determine the effect of various concentrations of DL-mevalonate-1-2-$^{14}C$ on the liver mince activity, several flasks each containing 3g of liver mince, 75 $\mu$moles of phosphate buffer pH 7 in a final volume of 5ml were incubated for 4 hours. Varying concentrations of DL-mevalonate-1-2-$^{14}C$ ($3.7 \times 10^4$ counts per mimute per labelled carbon) were added. The rate of $CO_2$ production turned to be linear up to $0.8 \times 10^{-3}$M (Fig.4).

Popjak (4) found that upon the addition of reducing agents, namely $(NAD^+)_+(NADH+H^+)$ and ascorbic acid to rat liver enzyme preparations , more of the NSL and carboxylic acids were formed at the expense of polyprenols. Subsequently, it became of interest to investigate the role of such agents in the synthesis of certain metabolites of mevalonic acid.

4. Effect of Ascorbic Acid: Initially the effect of ascorbic acid, neutralized to pH 7, was investigated at varying concentrations. (Fig.5) shows that at an optimal concentration of 0.03M, ascorbic acid stimulated the $CO_2$ production by 20%. Increasing the con-

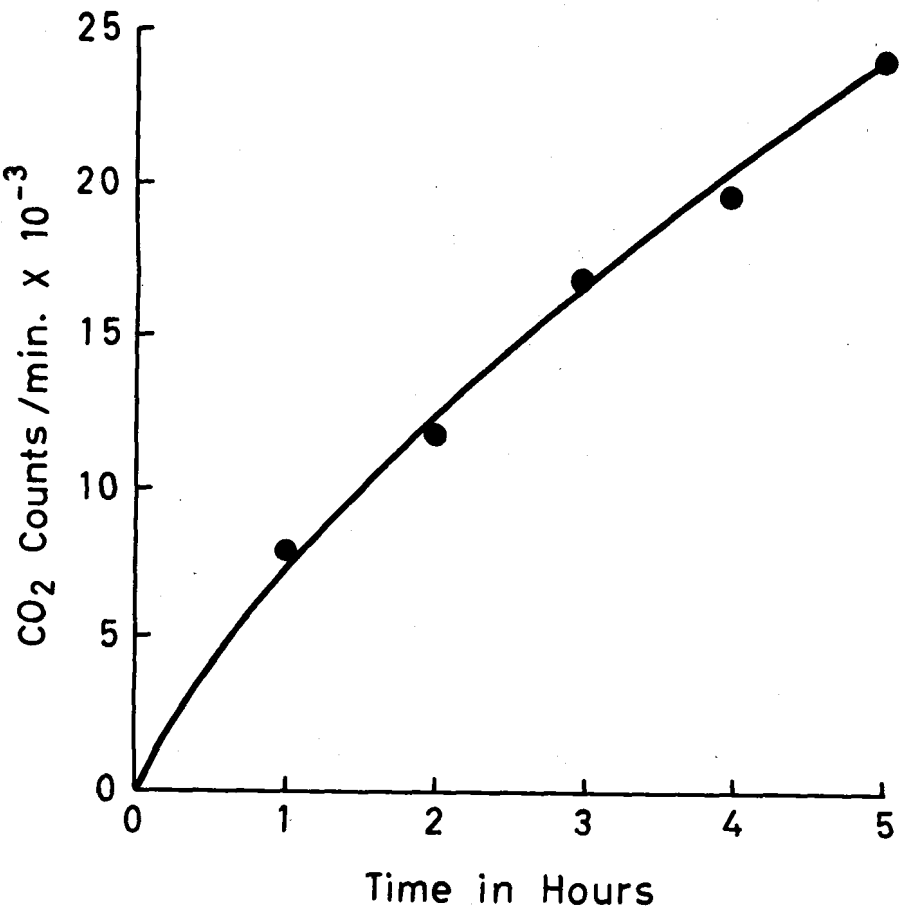



FIGURE 2. EFFECT OF TIME ON LIVER ACTIVITY

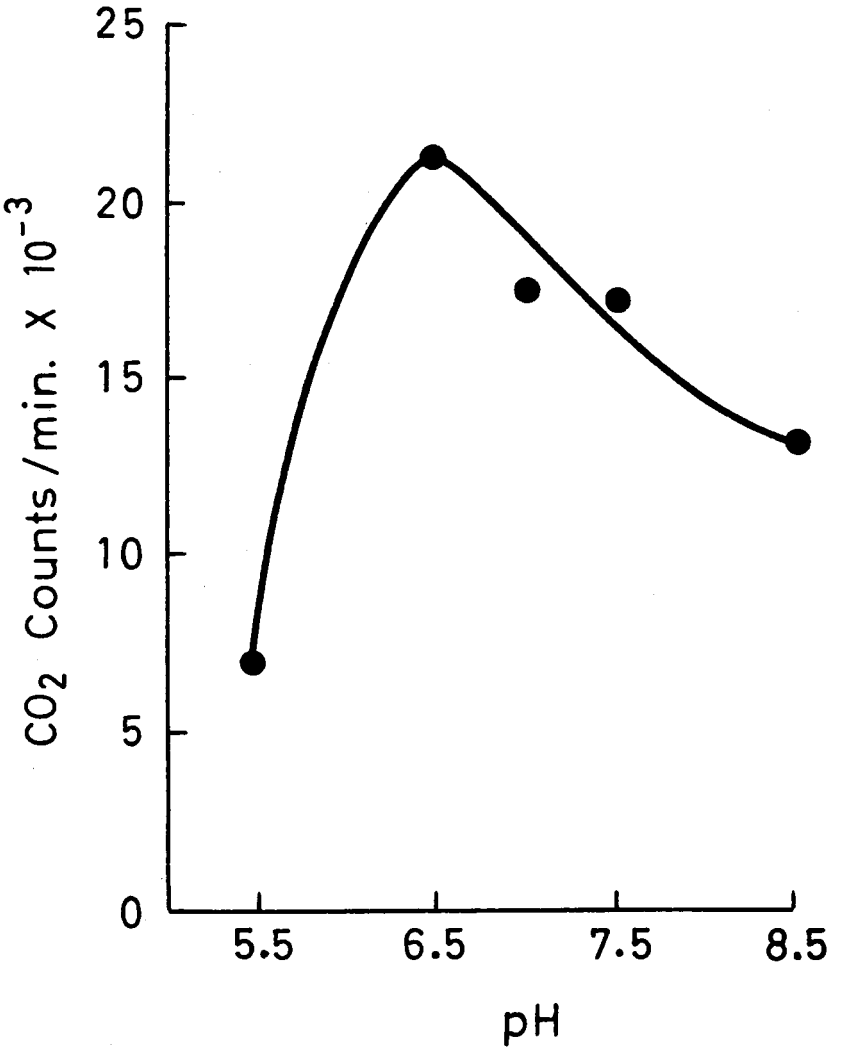



FIGURE 5. EFFECT OF pH ON LIVER ACTIVITY

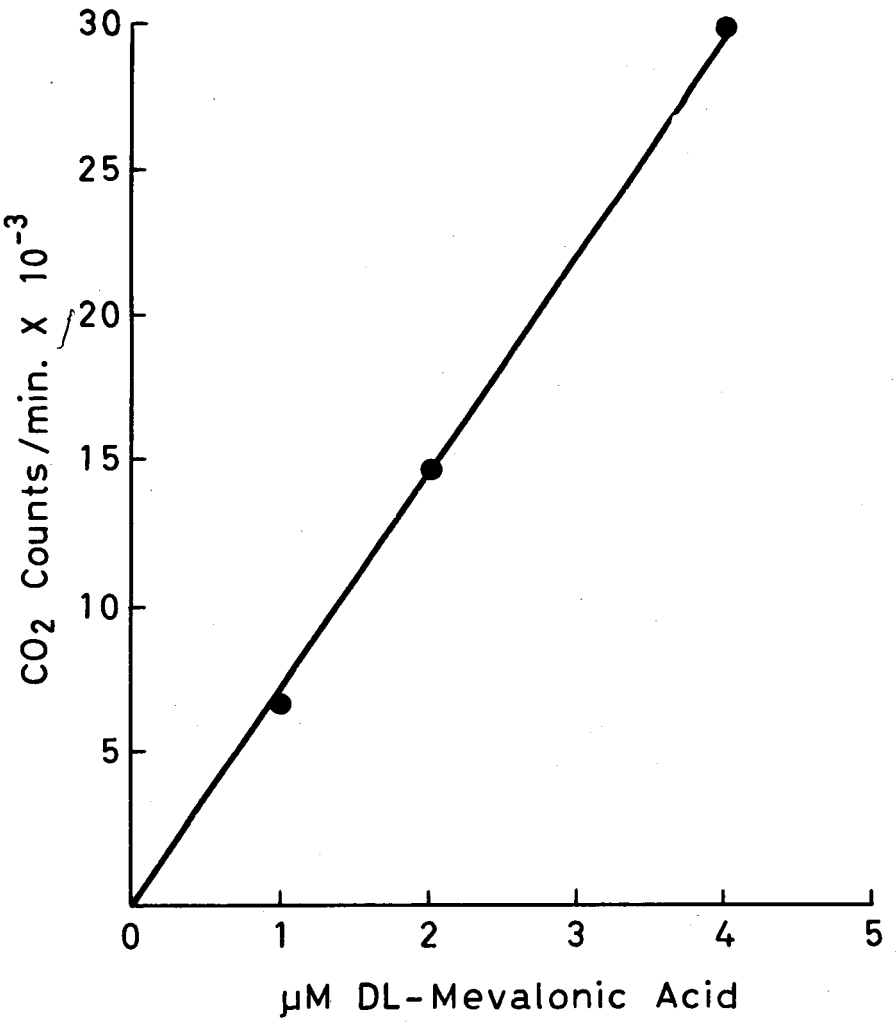



FIGURE 4. EFFECT OF MEVALONIC ACID CONCENTRATION ON LIVER ACTIVITY

centration of ascorbate beyond 0.05 caused a severe inhibition of the $CO_2$ liberated.

5. Effect of Reduced Glutathione: Another reducing agent was similarly tried in order to check upon the activity of liver mince. Reduced glutathione, GSH, was neutralized to pH 7 and added in varying concentrations. (Fig.6) shows that at an optimal concentration of 0.03M, GSH stimulated the $CO_2$ production by 70%. Increasing the concentration of GSH beyond 0.05M caused a severe inhibition of the $CO_2$ liberated.

The similarity in the patterns of both compounds namely ascorbic acid and GSH when used separately suggested a common site of action. Therefore the effect of both compounds together was investigated. (Fig.7) shows that optimal stimulation of 50% occurred when the concentration of each compound was approximately 0.015M. Increasing the concentration of each beyond 0.025M caused inhibition of the $CO_2$ liberated.

6. Effect of Cysteine and Mercaptoethanol: It became of interest to investigate oter thiols as well. When cysteine.HCl was similarly added to the liver incubation mixture, it was found that it acted as an inhibitor at all concentrations, (Fig.8).

It may be pointed out here that under the same conditions, mercaptoethanol acted as a strong inhibitor (table 2).

Different combinations of ascorbic acid, GSH, and cysteine were also added at varying concentrations to liver mince.

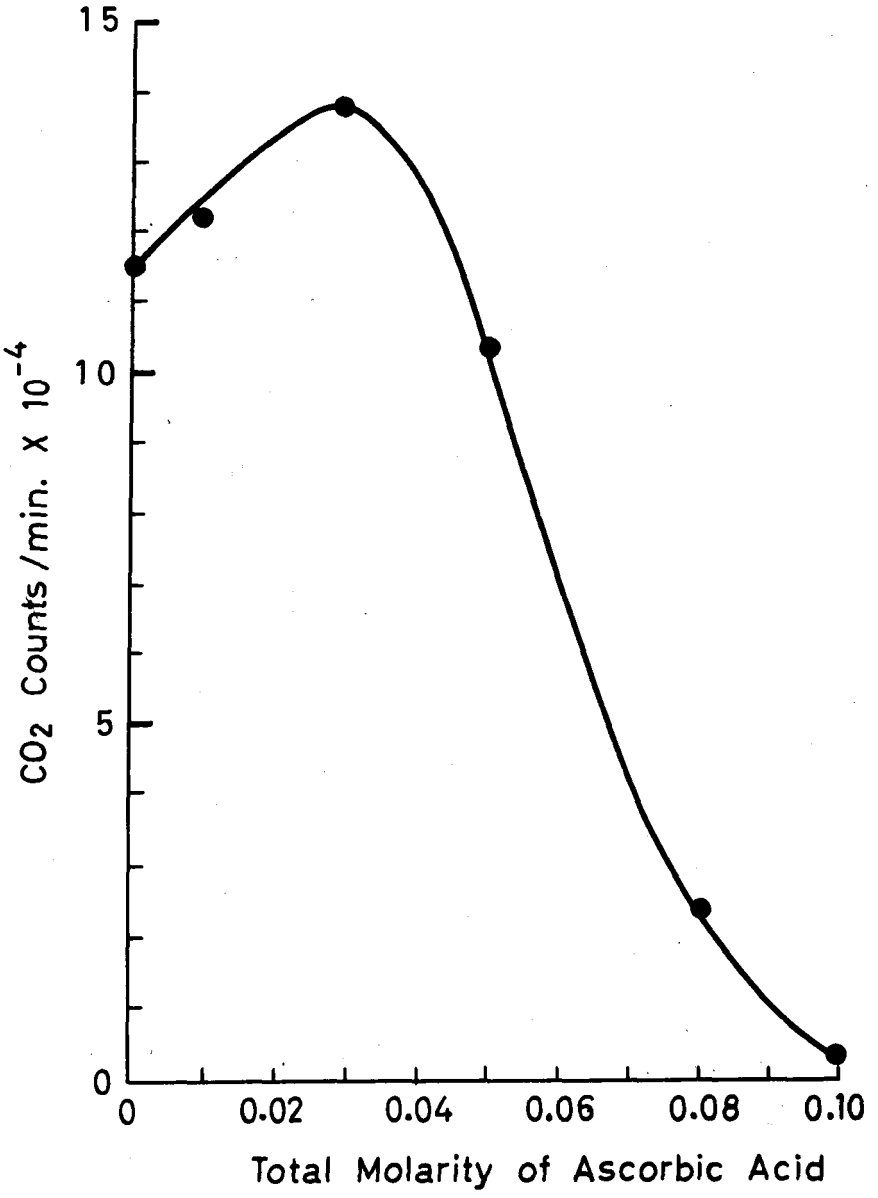



FIGURE 5. EFFECT OF ASCORBIC ACID ON LIVER ACTIVITY

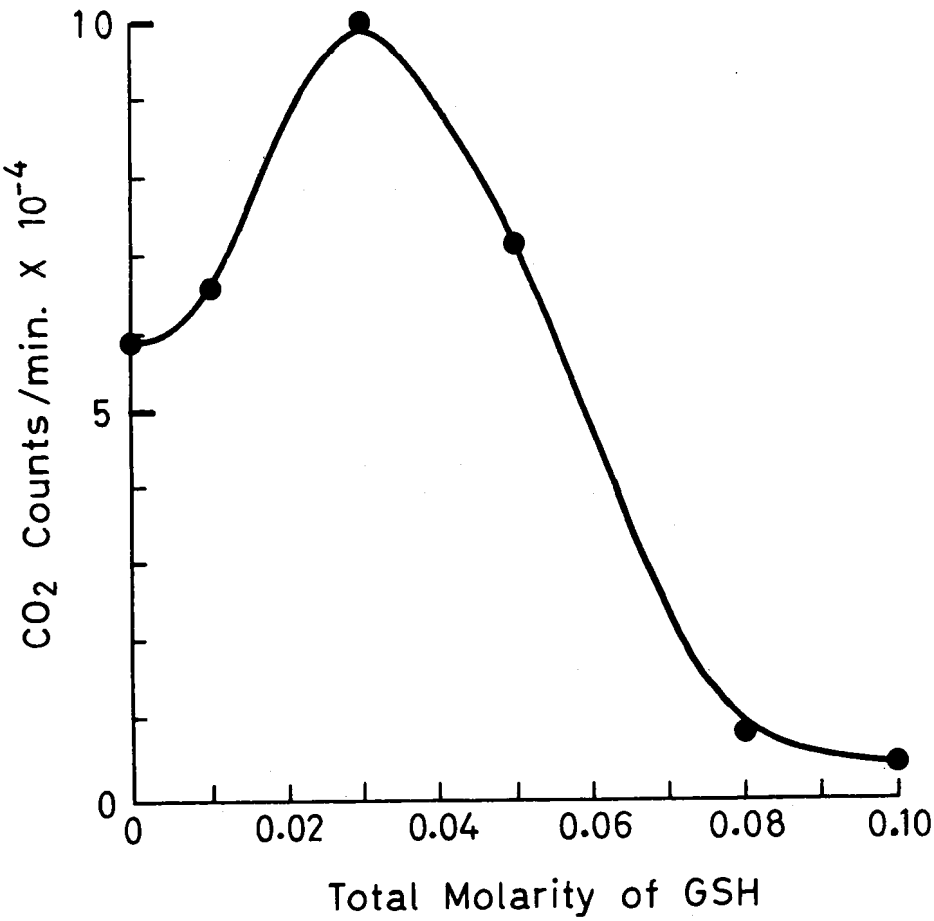



FIGURE 6. EFFECT OF GLUTATHIONE ON LIVER ACTIVITY

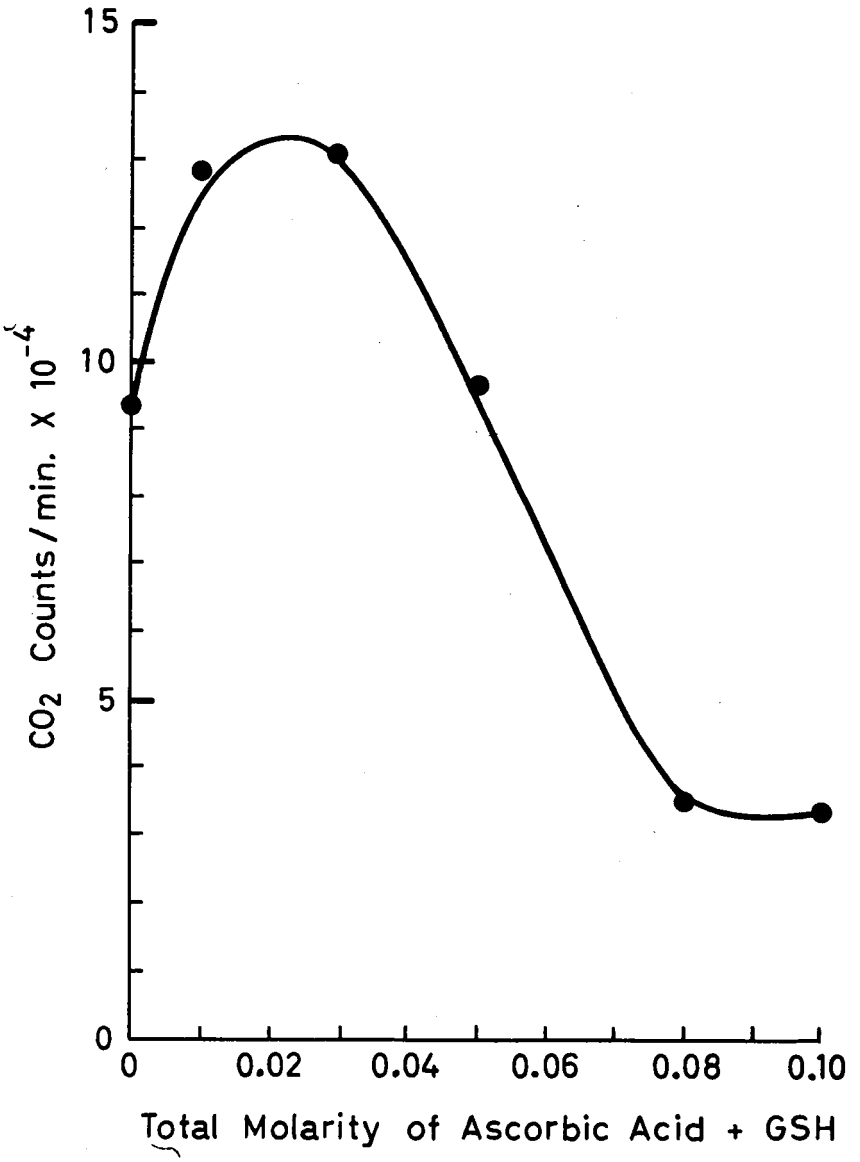



FIGURE 7. COMBINED EFFECT OF ASCORBATE + GSH ON LIVER ACTIVITY

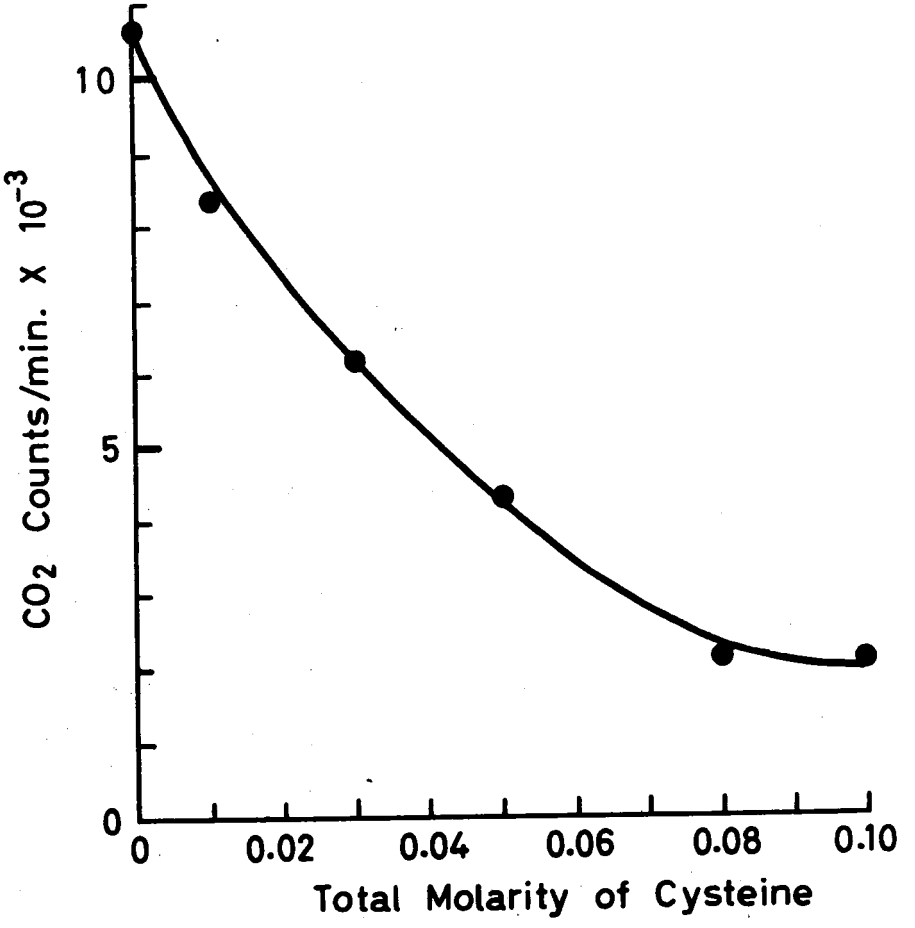



FIGURE 8. EFFECT OF CYSTEINE.HCl ON LIVER ACTIVITY

Table 2

**Effect of Mercaptoethanol on Liver Activity**

| Additions | $CO_2$ counts per minute $10^{-3}$ |
|---|---|
| Control | 24.3 |
| 0.03M GSH | 31.5 |
| 0.03M Mercaptoethanol | 5.4 |

Incubations were similarly carried and assayed. As indicated in (table 3), at 0.03M concentration ascorbic acid and GSH each separately acted as stimulator while cysteine acted as inhibitor. At higher concentrations all acted as inhibitors.

In (table 4) the first three flasks were kept at 0.03M concentrations and it is quite evident that the inhibition caused by cysteine is not reversed by the addition of stimulators namely ascorbate or GSH.It is to be noticed however that different degrees of stimulation and inhibition occurred in different incubations due to biological variations.

7. Effect of Anaerobiosis: It was found that anaerobiosis inhibited the activity of the liver mince. When nitrogen was used as the gas phase instead of air , the $CO_2$ liberated and non-saponifiable lipids synthesized were 40% of the aerobic incuation. It must be noted however , that there was no difference in the $CO_2$/NSL ratio whether under aerobic or anaerobic conditions.

8. Effect of Phenethyl Biguanide: After testing the inhibitory action of these different chemicals a known cholesterol inhibitor namely phenethyl biguanide was tested as well. It was found to inhibit $CO_2$ production at all concentrations (table 5). Therefore a cholesterol inhibitor blocked drastically the metabolism of mevalonate and this inhibition could not be reversed by the addition of GSH.

B-Extraction of the Metabolites of Mevalonic Acid: After studying the different optimal conditions for the metabolism of mevalonate by rat liver mince and the effect of the various chemicals, it

## Table 3

### Effect of Ascorbate, GSH, and Cysteine Each at 0.03 M On Liver Activity

| Additions | $CO_2$ Counts per minute x $10^{-3}$ |
|---|---|
| Control | 7.8 |
| 0.03 M Ascorbate | 27.0 |
| 0.03 M GSH | 23.0 |
| 0.03 M Cysteine | 3.8 |
| 0.03 M GSH + 0.03 M Ascorbate | 2.0 |
| 0.03 M GSH + 0.03 M Cysteine | 1.0 |

## Table 4

### The Combined Effect of Ascorbate, GSH, and Cysteine at 0.03 M On Liver Activity

| Additions | $CO_2$ Counts per minute x $10^{-3}$ |
|---|---|
| Control | 14.0 |
| 0.03 M Ascorbate | 40.1 |
| 0.03 M GSH | 34.0 |
| 0.005 M GSH + 0.025 M Cysteine | 14.5 |
| 0.005 M GSH + 0.025 M Ascorbate | 45.0 |
| 0.03 M GSH + 0.03 M Ascorbate | 4.0 |
| 0.03 M GSH + 0.03 M Cysteine | 2.0 |

Table 5

Effect of Phenethyl Biguanide on Liver Activity

| Additions | $CO_2$ Counts per minute x $10^{-3}$ |
|---|---|
| Control | 8.0 |
| 0.005 M P.B.G. | 1.2 |
| 0.01 M P.B.G. | 0.9 |
| 0.03 M GSH + 0.005 M P.B.G. | 1.0 |
| 0.03 M GSH + 0.01 M P.B.G. | 0.3 |

became of interest to investigate a rapid and quantitative procedure for the extraction of all the metabolites of mevalonate.

The classic procedures for the extraction of non-saponifiable lipids (11) extracted readily squalene, cholesterol among other related compounds which represent only one-third of the metabolites of mevalonate as shown here by the $CO_2/NSL \approx 3$.

A new method as described in the previous chapter was therefore followed. It provided the required stoichiometry between the carbon dioxide liberated and the mevalonate derived lipids. (Table 6) indicates that this method is a better approach to the study of the metabolites of mevalonate and offers a further evidence for the validity of using respired $CO_2$ as an assay for the metabolism of mevalonate.

C- Fractionation of the Metabolites of Mevalonic Acid:

1. Preliminary Chromatographic Resolution: Chromatography on a deactivated alumina column as previously described resolved the lipids derived from mevalonic acid into three major fractions with a total radioactivity of $6 \times 10^5$ counts per minute. (Figure 9) shows the distribution of radio-activity amongst the various fractions. (Table 7) indicates the percentage recovery of radioactivity in each fraction. The first fraction eluted with n-heptane was further purified while the remaining two fractions were saved.

## Table 6

## The Stoichiometric Relationship Between $CO_2$ Liberated from DL-Mevalonate-1-$^{14}C$ and the Metabolites of DL-Mevalonate-2-$^{14}C$

| Additions | | $CO_2$ Counts per minute x $10^{-3}$ | Metabolites Counts per minute x $10^{-3}$ |
|---|---|---|---|
| GSH | Control | 55.3 | 44.8 |
| | 0.03 M | 132.5 | 98.2 |
| | 0.10 M | 8.0 | 7.6 |
| Ascorbic Acid | Control | 45.1 | 42.1 |
| | 0.03 M | 71.7 | 58.6 |
| | 0.08 M | 24.1 | 23.1 |
| Ascorbic Acid + GSH | Control | 98.3 | 98.8 |
| | 0.01 M Asc.+0.01 M GSH | 118.0 | 110.0 |
| | 0.05 M Asc.+0.05 M GSH | 44.0 | 45.1 |
| Ascorbic Acid + GSH | Control | 94.0 | 80.6 |
| | .005 M Asc.+.005 M GSH | 116.0 | 93.1 |
| | .015 M Asc.+.015 M GSH | 133.1 | 98.7 |
| | 0.04 M Asc.+0.04 M GSH | 35.0 | 32.4 |
| Cysteine.HCl | Control | 10.8 | 10.2 |
| | 0.03 M | 5.3 | 5.6 |
| | 0.08 M | 3.1 | 3.2 |

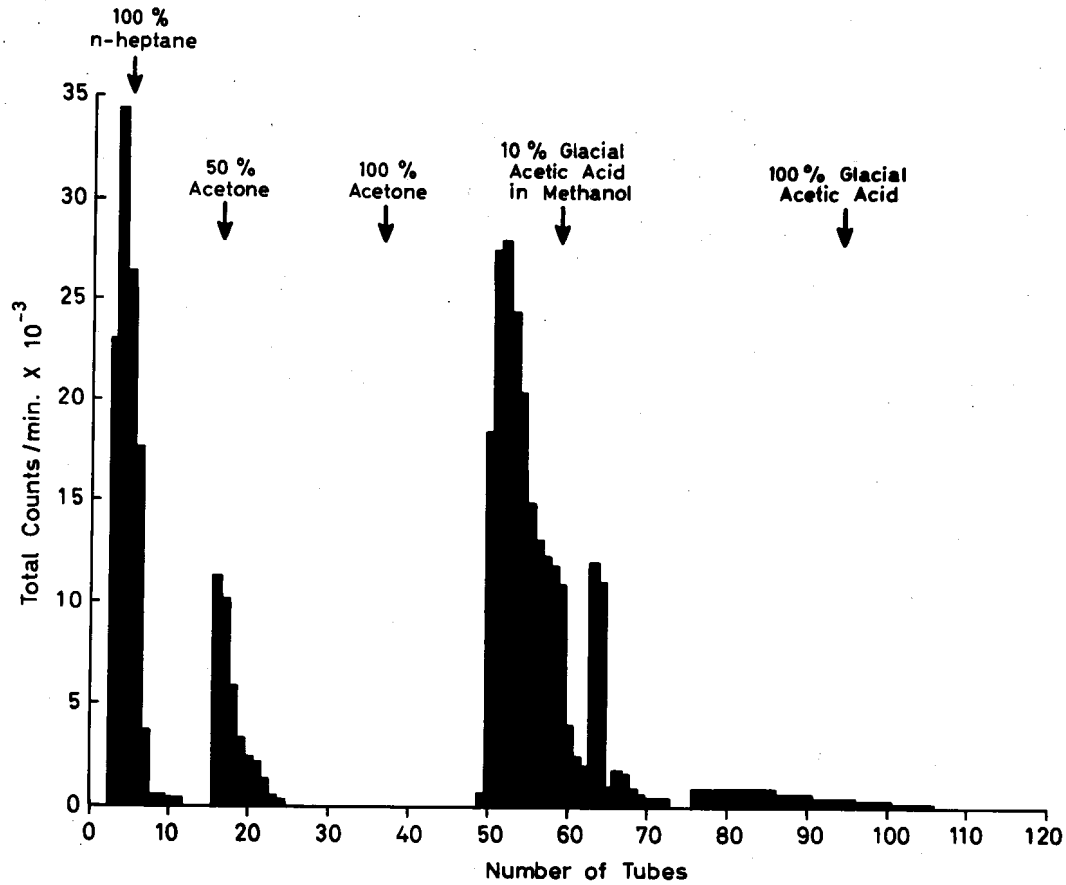



FIGURE 9. ELUTION PROFILE OF THE METABOLITES OF MEVALONATE ON DEACTIVATED ALUMINA COLUMN

Table 7

Chromatographic Resolution of Lipids Derived from DL-Mevalonate-1,2-$^{14}$C By Deactivated Alumina Column

| Solvent | No. of Tubes | Total Volume (ml) | Total Radio-Activity (cpm) | Percentage Recovery of Radioactivity |
|---|---|---|---|---|
| n-heptane | 13 | 250 | 107,600 | 18% |
| 50% Acetone-heptane | 14 | 280 | 36,500 | 6% |
| 10% Glacial HAc in Methanol | 29 | 600 | 228,100 | 38% |
| 50% Glacial HAc in Methanol | - | 200 | 17,500 | 3% |
| 100% Glacial HA | - | 400 | 14,500 | 2% |
| T O T A L | | | $4.0 \times 10^5$ | 67% |

2. Purification of the Heptane Fraction: Upon subjecting the first fraction eluted with n-heptane to thin layer chromatography it became evident that it represented a mixture of at least three different compounds. Subsequently, this fraction with total radioactivity of $1.1 \times 10^5$ counts per minute was further chromatographed on a silicic acid column as previously described and resolved into four major components. (Figure 10) shows the distribution of radioactivity amongst the various fractions. (Table 8) indicates the percentage recovery of radioactivity in each fraction.

The first and major component eluted with n-heptane decomposed and could not be purified any further. Repeated attempts to resolve and purify the decomposed products by column chromatography were not successful. The remaining three fractions were subjected to thin layer chromatography. More than one component was present in all but apparently the second fraction eluted with 1% (V/V) ether in heptane was the purest. It was called $X_1$ and chosen for further investigation while the other two were saved.

3. Isolation of a $C_{16}$-Keto Acid: $X_1$ with total radioactivity of $1.1 \times 10^4$ counts per minute was further purified by ethanol-water precipitation as

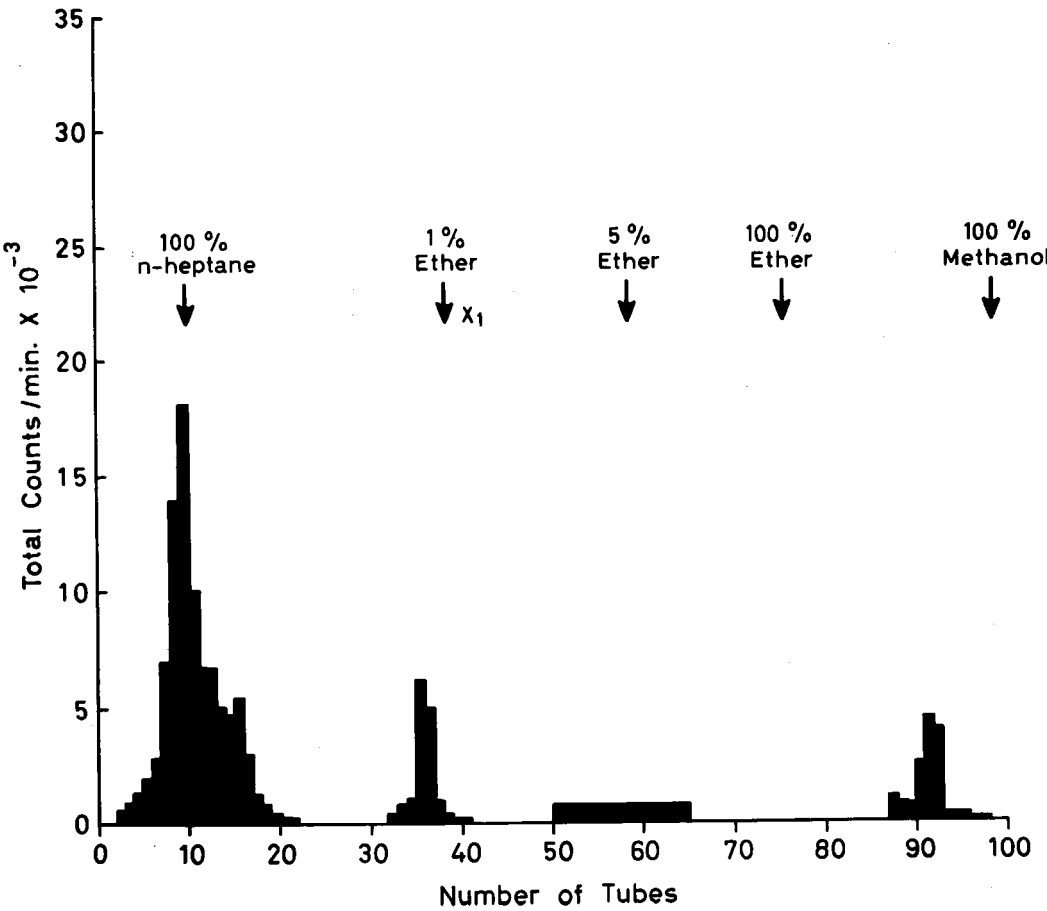



**FIGURE 10. ELUTION PROFILE OF THE HEPTANE FRACTION ON SILICIC ACID COLUMN**

Table 8

Chromatographic Resolution of n-Heptane Fraction By Silicic Acid Column

| Solvent | No. of Tubes | Total Volume (ml) | Total Radio-Activity (cpm) | Percentage Recovery of Radioactivity |
|---|---|---|---|---|
| n-heptane | 30 | 500 | 67,300 | 63% |
| 1% Ether | 30 | 500 | 11,300 | 10% |
| 5% Ether | 25 | 500 | 18,600 | 17% |
| Methanol | 40 | 800 | 10,400 | 9% |
| T O T A L | | | $1.1 \times 10^{5}$ | 99% |

previously mentioned then applied on a silicic acid column. (Fig.11) shows that $X_1$ was resolved into two components. (Table9) indicates the percentage recovery of radioactivity in each fraction.

The first and major component, called $X_2$, eluted with 1% (V/V) ethyl acetate in heptane was further purified while the second fraction eluted with pure methanol was saved. $X_2$ was recrystallized from hot 95% ethanol to a constant specific activity of 18 counts per minute per mg as shown in (table 10). Then it was rechecked for purity by thin layer chromatography using six different solvents as previously mentioned. In all cases thin layer chromatography of $X_2$ gave a single spot. The $R_f$ values are shown in (table 11).

D-Physical Properties of $X_2$:

$X_2$ is a white solid M.P. 49-51°C with needle-shaped crystals, when crystallized from 95% ethanol, and a waxy texture.

$X_2$ is insoluble in water, dilute alkali, $NaHCO_3$, or dilute acids. It is soluble in organic solvents and concentrated sulphuric acid.

E-Chemical Properties of $X_2$:

1. Elementary Analysis: Microanalyses of $X_2$ dried at 50°C and high vacuum as previously mentioned reported different data from different laboratories as indicated in (table 12)

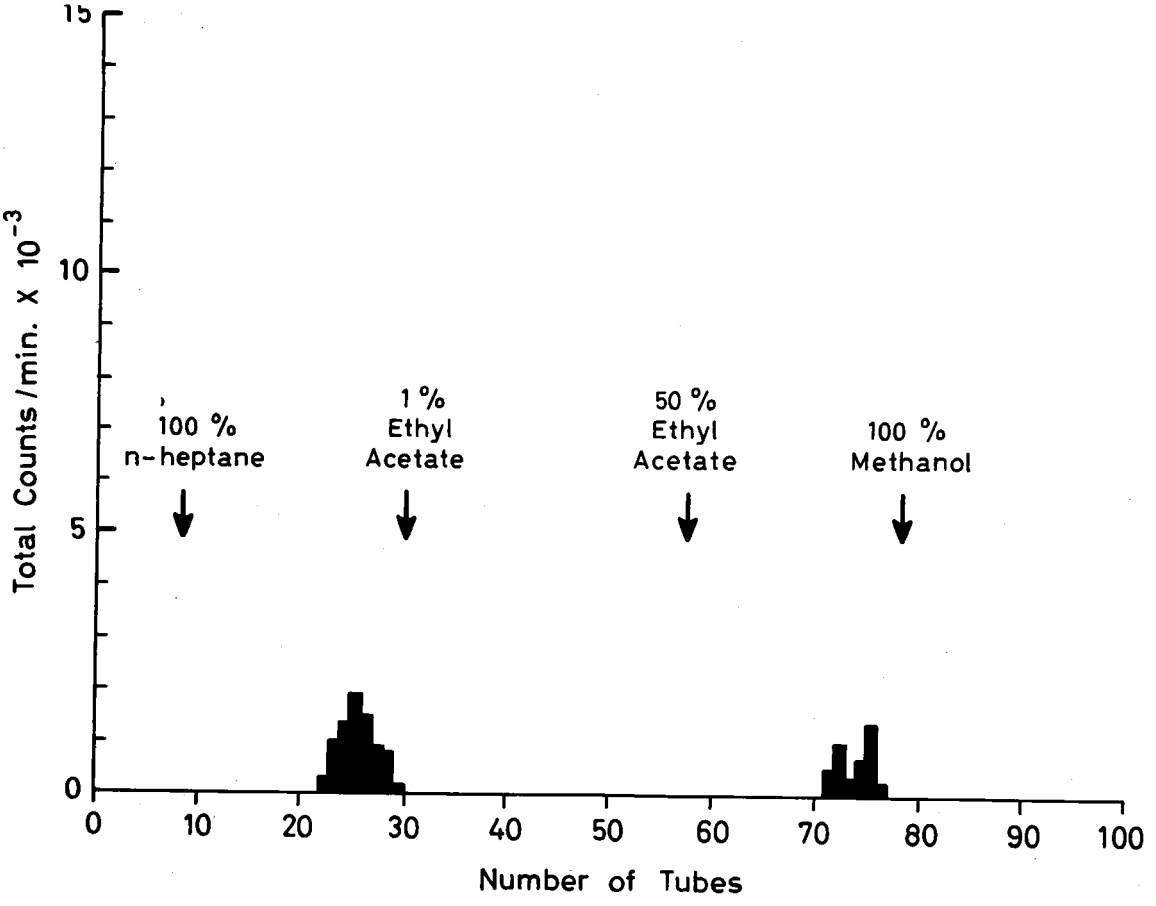



FIGURE 11. ELUTION PROFILE FOR OBTAINING $X_2$ BY SILICIC ACID COLUMN

Table 9

Chromatographic Resolution of 1% Diethyl Ether in Heptane Fraction by Silicic Acid Column

| Solvent | No. of Tubes | Total Volume (ml) | Total Radio-Activity (cpm) | Percentage Recovery of Radioactivity |
|---|---|---|---|---|
| 1% Ethyl Acetate in heptane | 25 | 300 | 6,900 | 61% |
| 100% Methanol | 30 | 350 | 4,100 | 36% |
| T O T A L | - | - | $1.1 \times 10^4$ | 97% |

Table 10

Recrystallization of $X_2$ to a Constant Specific Activity

| No. of Trials | Melting Point °C | Specific Activity cpm/mg |
|---|---|---|
| 1 | 48-50 | 21 |
| 2 | 49-51 | 18 |
| 3 | 49-51 | 18 |

## Table 11

### The $R_f$ Values Obtained in Thin Layer Chromatography of $X_2$ and its Derivatives

| Sample | n-Hexane | Ethyl Acetate Hexane | Hexane-Ether HAc-Methanol | Hexane-Ether $NH_4OH$-Methanol | $CHCl_3$-Acetone | $CH_2Cl_2$ |
|---|---|---|---|---|---|---|
| $X_2$ | 0 | 0.21 | 0.38 | 0 | 0.24 | 0.10 |
| Methyl Ester of $X_2$ | 0 | 0.87 | 0.85 | 0.92 | 0.83 | 0.91 |
| 2,4-DNP Hydrazone of $X_2$ | 0 | 0.50 | 0.36 | 0.41 | 0.85 | 0.79 |

## Table 12

## Elementary Chemical Analysis of $X_2$

| Type of data | %C | %H | %O | %$-CH_3$ | Mol.Wt. | Formula |
|---|---|---|---|---|---|---|
| Reported[1] | 75.02 | 12.09 | 12.43 | 2.86 | 270 | - |
| Possible Values | 71.64 | 10.44 | 17.91 | - | 268 | $C_{16}H_{28}O_3$ |
| | 71.11 | 11.11 | 17.78 | - | 270 | $C_{16}H_{30}O_3$ |
| | 76.12 | 11.94 | 11.94 | - | 268 | $C_{17}H_{32}O_2$ |
| | 75.56 | 12.59 | 11.85 | - | 270 | $C_{17}H_{34}O_2$ |
| Reported[2] | 74.89 | 12.02 | 12.51 | 7.12 | 337 | - |
| Possible Values | 73.96 | 11.24 | 14.24 | - | 338 | $C_{21}H_{38}O_3$ |
| | 74.12 | 11.76 | 10.88 | - | 340 | $C_{21}H_{40}O_3$ |

1. Reported by Dr. Albert Bernhardt Microanalystisches Laboratorium, 433 Muhleim , Germany.

2.

Also reported by this laboratory a hydrogenation value of 0.4 moles $H_2$ per mole of $X_2$.

2. Absorption Spectrum: $X_2$ absorbs in ultra-violet at 230 mμ then with vaguely defined absorption bands at 270 mμ and 280 mμ (Fig.12). The solvent used was ethanol as previously mentioned.

3. Infrared Spectrum: IR spectrum of $X_2$ was determined as previously described, the solvent used was chloroform. (Fig.13) indicates peak maxima at 3540 $cm^{-1}$; 3120 $cm^{-1}$; 2930 $cm^{-1}$; 2880 $cm^{-1}$; 2680 $cm^{-1}$; 1730 $cm^{-1}$; 1720 $cm^{-1}$; 1470 $cm^{-1}$; 1415 $cm^{-1}$; 1290 $cm^{-1}$; 1120 $cm^{-1}$; and 940 $cm^{-1}$.

4. Methyl Ester of $X_2$: $X_2$ was esterified as previously mentioned. The methyl ester of $X_2$ was checked for purity by thin layer chromatography in six different solvents. In all cases thin layer chromatography of $X_2$ methyl ester gave a single spot. The $R_f$ values are shown in (table 11).

$X_2$ methyl ester was subjected to gas-liquid chromatography as previously described. Standard methyl stearic acid and methyl palmitic acid were tested under the same conditions. The peak registered for $X_2$ methyl ester coincided more with that of methyl palmitic acid (Fig.14).

Infrared spectrum of $X_2$ methyl ester was obtained as well, it was found to be similar to that of methyl palmitate.

5. Derivative Formation: 2,4-Dinitrophenyl hydrazine derivative of $X_2$ was prepared as previously mentioned. The hydrazone of $X_2$ was tested for purity by thin layer chromatography in six different solvents. In all cases a single spot was apparent. The $R_f$ values are shown in (table 11).

The absorption spectrum of $X_2$ 2,4-dinitrophenyl

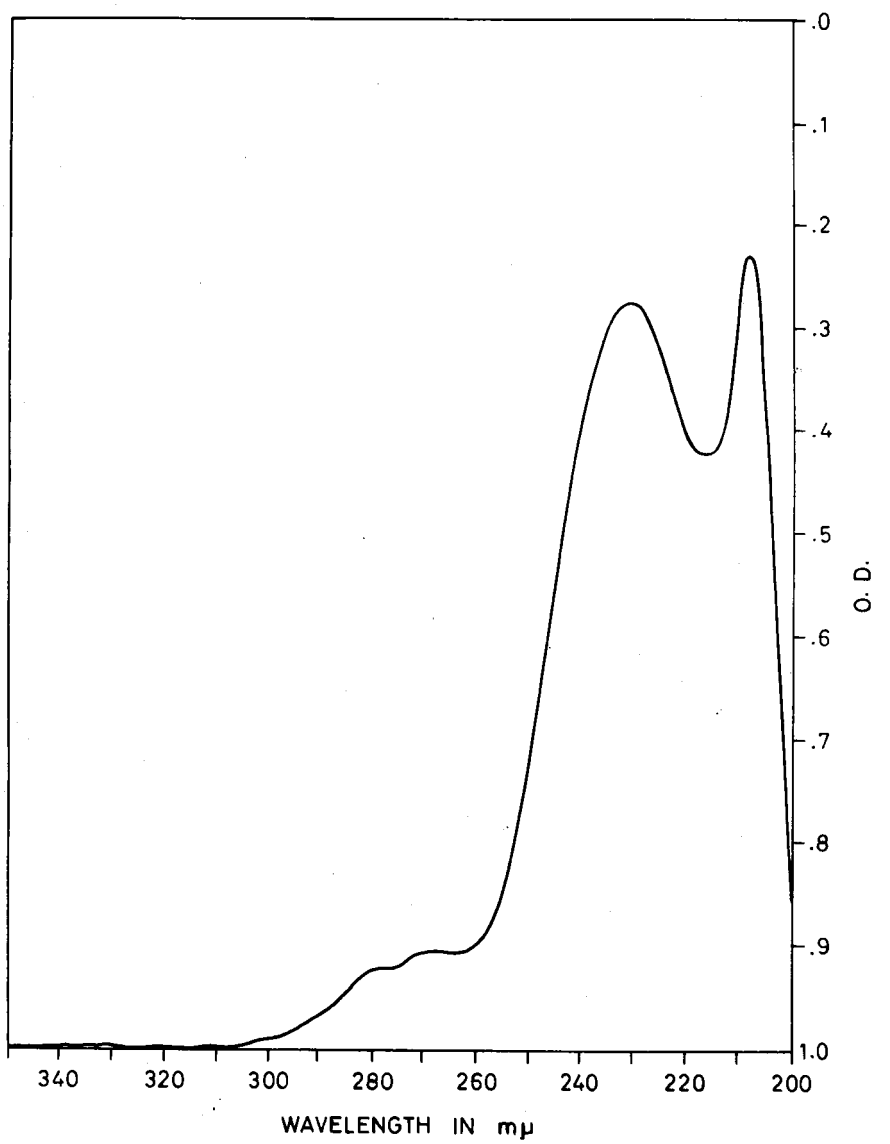



FIGURE 12. ABSORPTION SPECTRUM OF $X_2$ IN ETHANOL

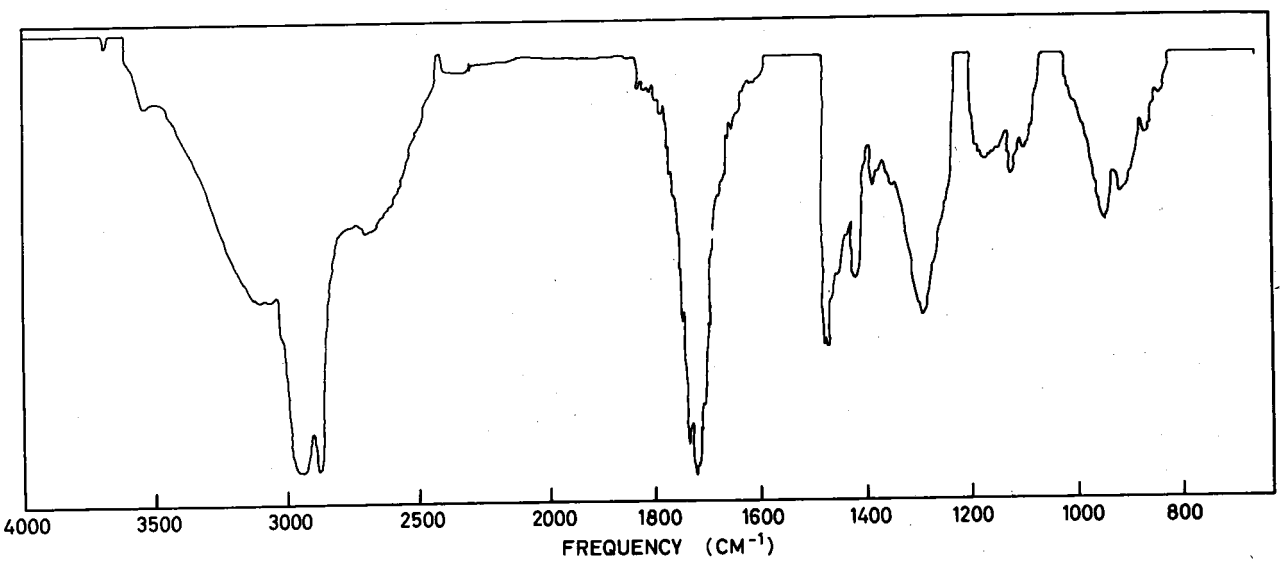



FIGURE 13. INFRA-RED SPECTRUM OF $X_2$ IN CHLOROFORM

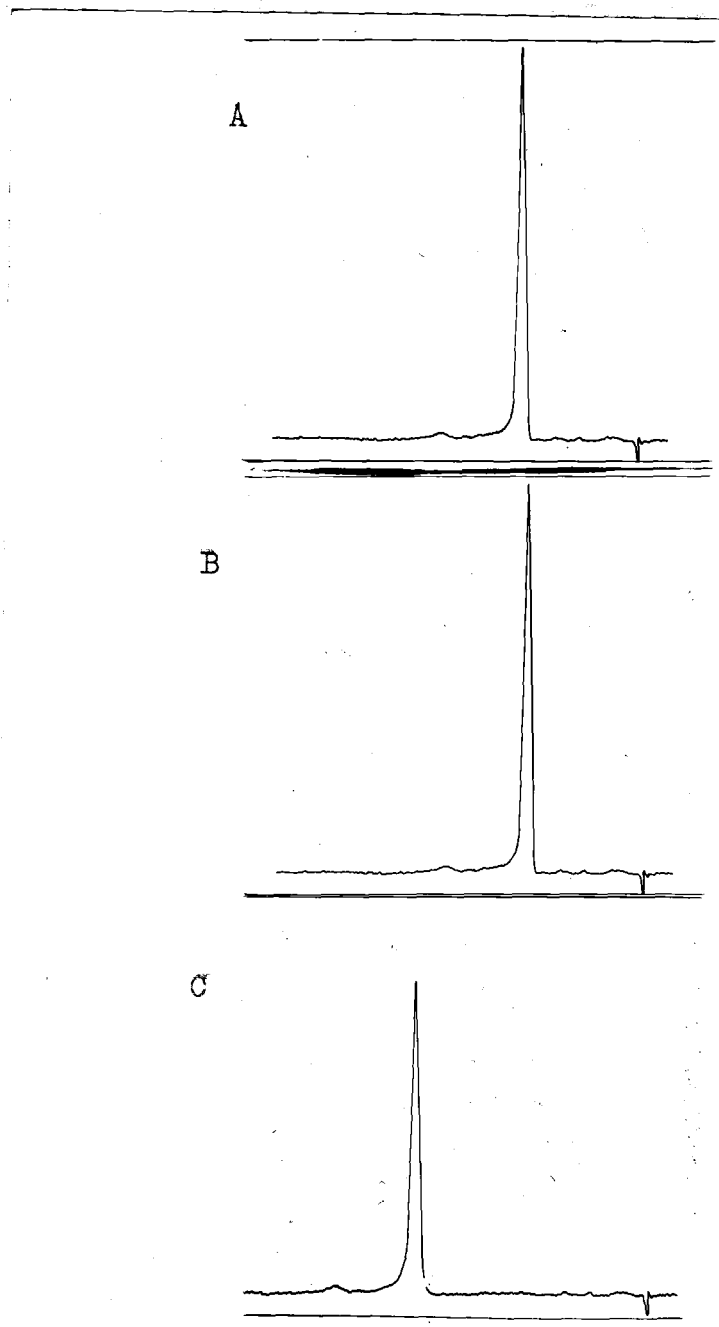



FIGURE 14. PEAKS REGISTERED BY GAS-LIQUID CHROMATOGRAM.

A- represents $X_2$ methyl ester

B- represents methyl palmitate

C- represents methyl stearate

hydrazone was obtained as previously mentioned. The solvent used was 95% ethanol. (Fig.15) indicates absorption bands at 228 mμ; 260 mμ; and 360 mμ.

6. Nuclear Magnetic Resonance: The n.m.r. spectrum of $X_2$ (Fig.16) showed a triplet centered at $\tau$= 4.67(1H); a doublet centered at $\tau$= 7.68(1H); a large singlet at $\tau$=8.73(28H); and another singlet at $\tau$=9.10(3H).

7. Chemical Tests: $Br_2$ water and $KMnO_4$ solution were used to test for unsaturation in $X_2$. Both tests gave negative results.

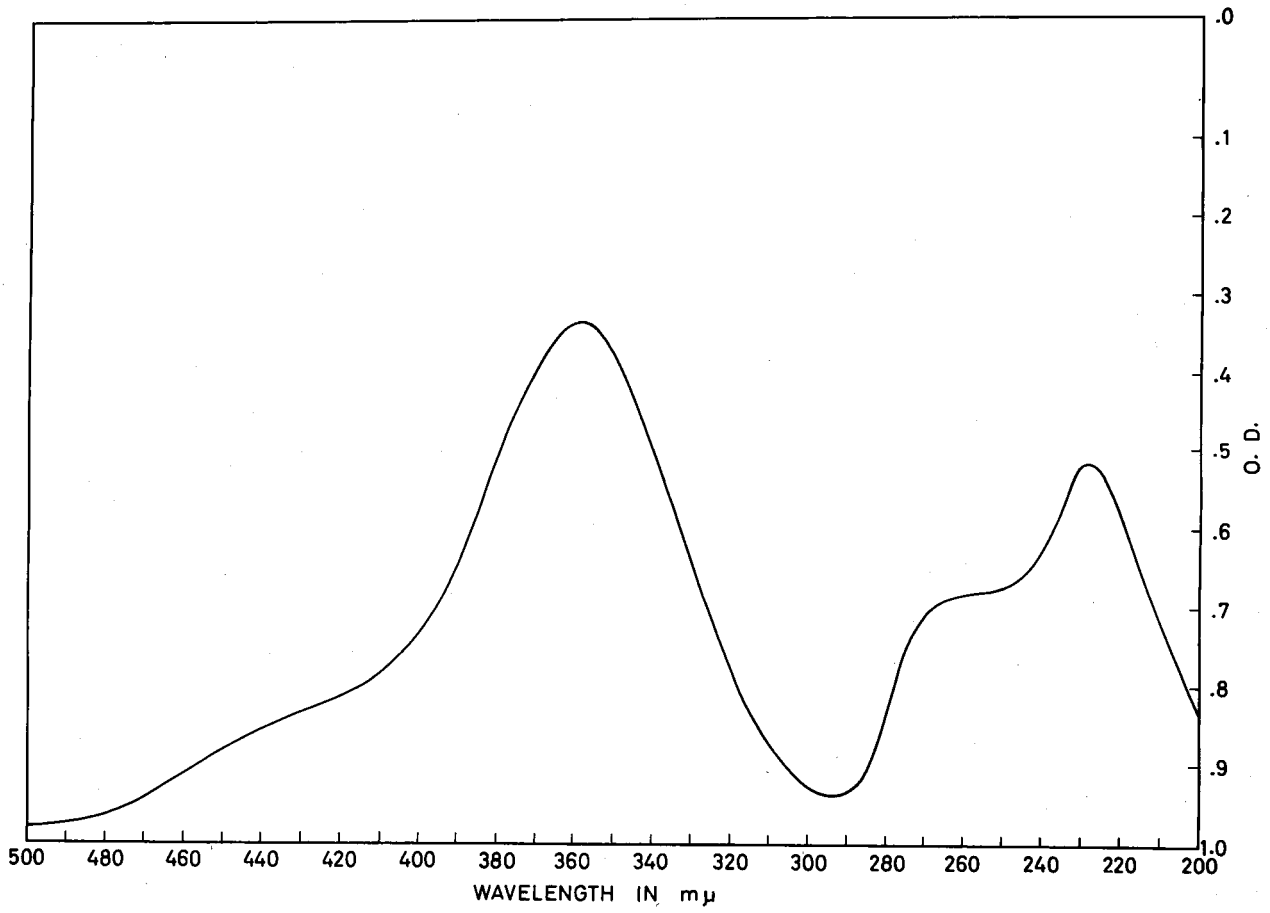
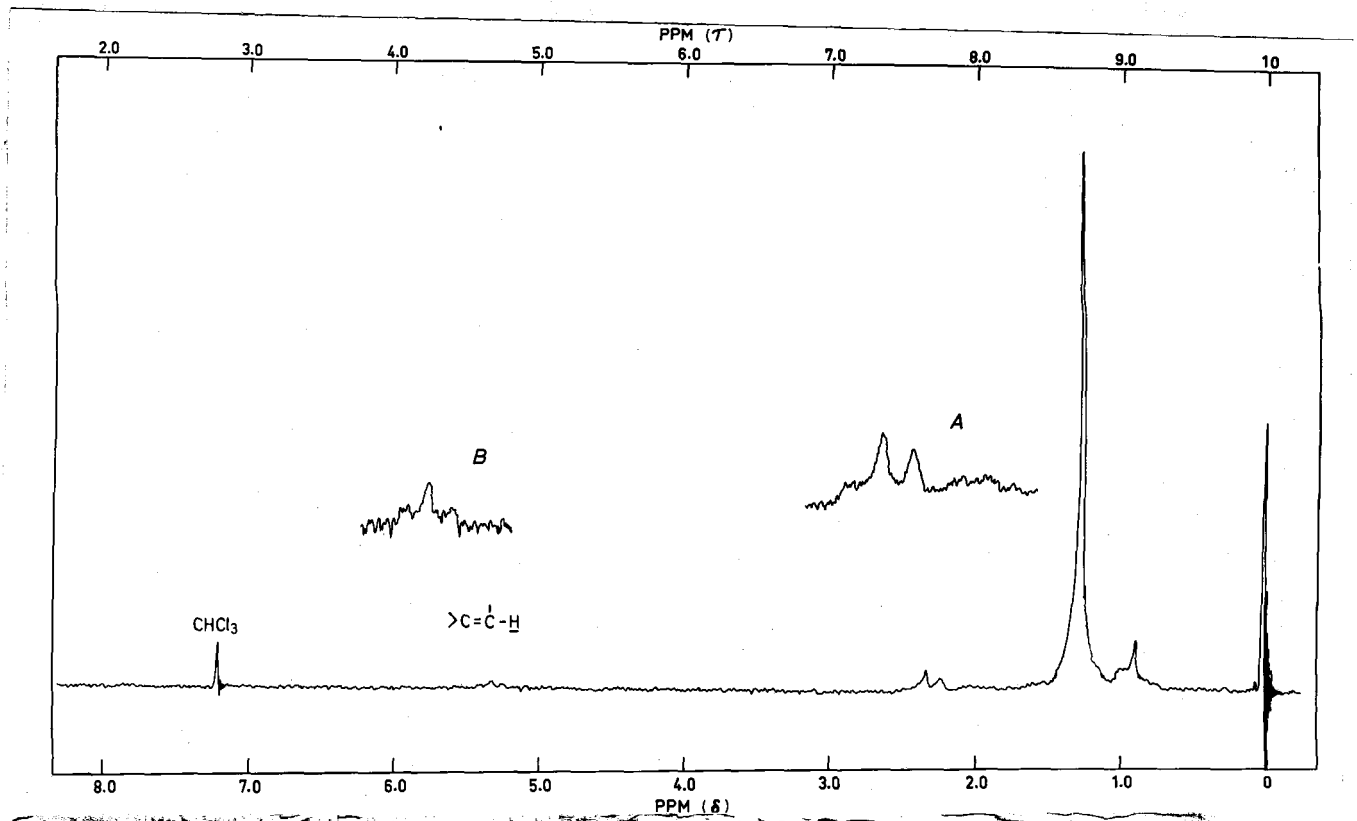



FIGURE 15. ABSORPTION SPECTRUM OF 2, 4 - DINITROPHENYL HYDRAZINE DERIVATIVE OF $X_2$ IN ETHANOL



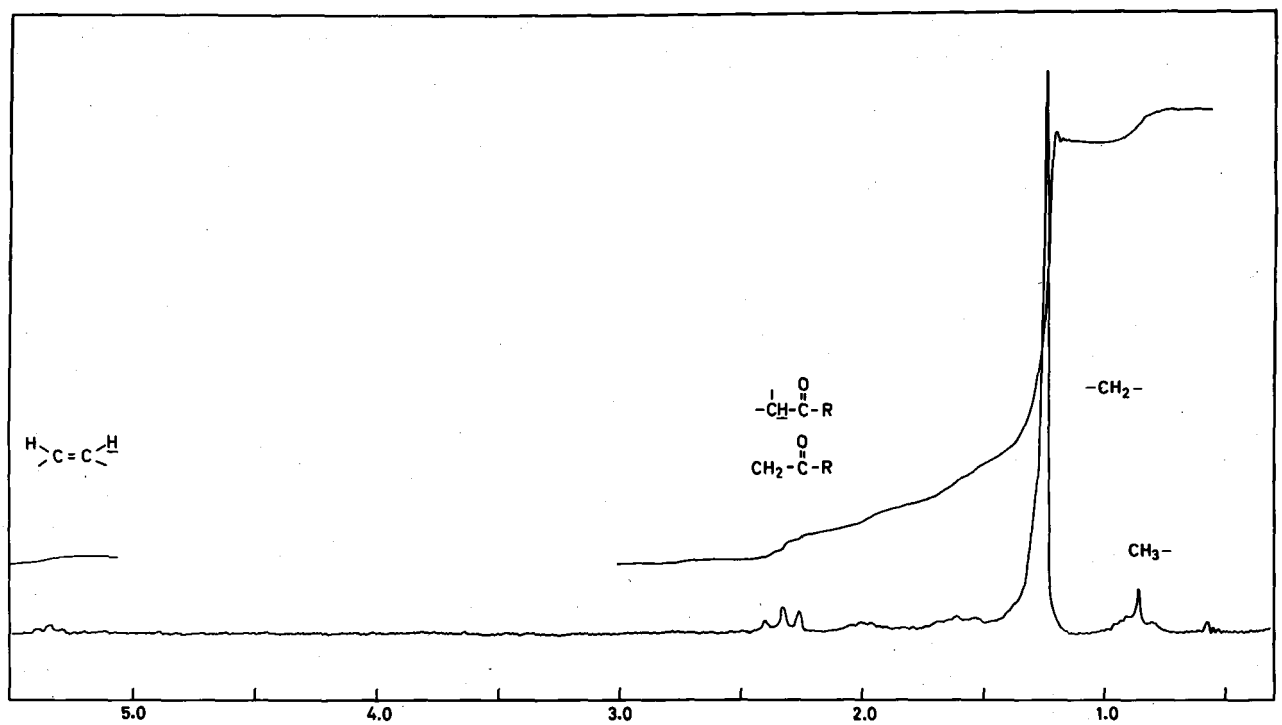




FIGURE 16. THE N.M.R. SPECTRUM OF $X_2$ IN $CDCl_3$

# CHAPTER IV

## DISCUSSION

Popjak (4) found that when an enzymatic system of rat liver homogenate was first incubated on a large scale for 2 hours with DL-mevalonate-2-$^{14}$C, about 25 percent of the DL-mevalonate added was converted into the polyprenol derivatives; also a certain amount of acidic products was formed. Accordingly he tested several samples of the preliminary bulk of incubation in different ways. He found that the addition of microsomes, ascorbic acid and $(NAD^+)+(NADH+H^+)$ then further incubation resulted in the formation of squalene, cholesterol and carboxylic acids at the expense of the polyprenols. Further analysis proved that the farnesol and nerolidol components of the polyprenol mixture were principally involved in the change.

Another point was noticed namely in the absence of either $(NAD^+)+(NADH+H^+)$ or ascorbic acid very little squalene and sterols was formed; the polyprenols were converted into carboxylic acids which suggested a catabolic disposal of squalene precursors by a mechanism not yet determined. Popjak also found that these carboxylic acids are not intermediates in squalene biosynthesis because when they were allowed to accumulate in an incubation medium the subsequent addition of $(NAD^+)+(NADH+H^+)$ and ascorbic acid failed to cause the synthesis of squalene and cholesterol. These

carboxylic acids therefore appear to inhibit the utilization of the polyprenol derivatives for the synthesis of squalene.

In view of these findings, it became of interest to isolate some of these intermediates for the purpose of identification and elucidation of the biochemical mechanisms involved in the catabolic disposal of squalene precursors and such transformations.

Prior to this study, it was decided to explore possible agents which stimulate the production of the neutral and acidic polyprenols. Reducing agents namely ascorbic acid and GSH were added each individually and with varying concentrations to the incubation media. In each case stimulation at a low concentration of 0.03 M was recorded; drastic inhibition occurred at higher concentrations of 0.08 M and beyond. It may be noted here that thiols like cysteine and mercaptoethanol when added under similar experimental conditions proved to be inhibitory at all concentrations.

These findings as established in the results suggest the presence of a redox system in operation whereby $(NAD^+)+(NADH+H^+)$, ascorbic acid, or GSH are affecting similarly the same site. This redox system does not involve the activity of a sulphydril group because as already mentioned when cysteine or mercaptoethanol were similarly added they proved to be inhibitory at all concentrations.

Furthermore anaerobic conditions also inhibited the

production of these compounds. Oxygen was needed to stimulate the formation of these unknowns. The role of this redox mechanism, if confirmed, in the synthesis of sterols and terpenes is not known. It might be noted as well here that upon the addition of a known cholesterol inhibitor, namely phenethyl biguanide, to the incubation medium it acted as an inhibitor as well to these unknown substances. Therefore, the factors which stimulate squalene and cholesterol production stimulate as well the formation of these unknowns and the factors which inhibit squalene and cholesterol formation act too as inhibitors to these unknowns. This might indicate that the two mechanisms in operation, the first leading to squalene and cholesterol formation and the second leading to the production of certain unknown substances, are acting simultaneously. Upon the accumulation of too much intermediates in the first, the second mechanism would operate to relieve the tension.

The major difficulty encountered in assaying for the polyprenes, excluding squalene and cholesterol and their immediate intermediates, was the absence of a method of assay and quantitative isolation of these metabolites. The activity of the carbon dioxide liberated from DL-mevalonic acid-1-$^{14}$C lactone was used as the assay system. It was therefore important to establish a stoichiometric relationship between the carbon dioxide liberated and the metabolites of mevalonic acid in the liver.

Accordingly, a good method for the extraction of the metabolites of mevalonate was required. The classical methods for extracting the lipids involved:

A. Saponification of the incubation mixture.

B. Extraction with petroleum ether under alkaline conditions.

C. Measurement of the activity of the petroleum ether and subsequent resolution of the non-saponifiable lipids by chromatography.

D. Extraction of the aqueous layer with petroleum ether under acidic conditions.

E. Measurement of the activity of the petroleum ether layer and its resolution by chromatography.

Such a method was inadequate and tedious. It offered drastic conditions for extraction whereby all compounds were thus classified as strictly alkaline or acidic. It was modified and a second method was used which involved the first four steps then the neutralization of the petroleum ether layer with 0.1 N $KHCO_3$ and subsequent extraction of the $KHCO_3$ layer with petroleum ether under acidic conditions.

In this method extraction under milder conditions gave the opportunity for the neutral compounds to be separated on one side rather than be placed under strictly acidic or basic conditions.

However, such methods were far from offering the required stoichiometry between the carbon dioxide liberated

and metabolites of mevalonate. Heavy contamination with unreacted mevalonic acid was confronted as well. A further improvement yielded a third procedure which was outlined in the chapter of methods.

It is worth noticing that all of these methods involved the acidification of the water layer and subsequent extraction with petroleum ether after the initial extraction under alkaline conditions. Such a procedure was followed in order to recover some of the neutral and acidic metabolites. This is in accordance with Popjak's findings (4) that most of the petroleum ether-soluble substances were neutral in character and released after acidification. However, a quantitative recovery was difficult because of the limited solubility of these compounds in petroleum ether. In addition, contamination with residual radioactive mevalonate in the aqueous layer was unavoidable.

A fourth method was later followed; it involved:

A. Saponification of the incubation mixture.
B. Saturation of the saponified mixture with sodium chloride.
C. Extraction with acetone: petroleum ether 7:3 (V/V) under alkaline conditions.
D. Extraction of the petroleum ether-acetone layer with acetone.
E. Measurement of the activity of the acetone layer and its resolution by chromatography.

All extractions were rapidly accomplished and a minimum loss of

radioactivity was encountered because of such a relatively short procedure. Furthermore, this method provided:

1- The required stoichiometry between the carbon dioxide liberated and the mevalonate derived lipids.

2- A reliable approach and estimate of the compounds that are derived from mevalonate.

3- No contamination with residual -$^{14}$C-mevalonate.

4- A further evidence for the validity of using respired carbon dioxide as an assay for the metabolism of mevalonate by liver tissues.

Following such an extraction procedure, different compounds representing the metabolites of mevalonate were resolved by chromatography. Although a number of these metabolites was isolated, a definitive structural study was precluded because of their instability upon purification. However , during the course of purification a crystalline product, $X_2$, accompanied the oily metabolites was isolated. Although it did not possess a considerably high specific activity comparable to the other metabolites, it was decided to explore its structure because of its relative stability and purity.

The purity of $X_2$ was evident in different items:

1- Thin layer chromatography of $X_2$ in six different solvents and at varying concentrations, least of which was 0.25$\mu$g, gave a single spot(table 11).

2- The methyl ester of $X_2$,when subjected to gas-liquid chromatography, was resolved as a single sharp peak (Fig.14).

3- The 2,4-dinitrophenyl hydrazone of $X_2$ yielded a single spot in thin layer chromatography using six different solvents.

4- $X_2$ was recrystallized three times to a constant specific activity of 18 counts per minute per mg.

5- $X_2$ was recrystallized to a constant melting point of 49-51°C

6- The infrared spectrum of $X_2$ yielded sharp peaks characteristic of a pure compound.

$X_2$ has been identified as a keto acid with an empirical formula $C_{16}H_{30}O_3$ and a molecular weight of 270±2. Various analyses were performed on $X_2$ to determine its functional groups as previously reported. Itwas scanned in the infrared region of the spectrum (Fig. 13). The presence of a band at 3540 $cm^{-1}$ suggests the -O-H stretch of carboxylic acids. The wide band between 3120-2680 $cm^{-1}$ represents the -C=OH group. The strong double peak at 1730 $cm^{-1}$ and 1720 $cm^{-1}$ indicates the C=O stretch of a ketone (17). No peak was registered between 1670-1600 $cm^{-1}$ characteristic of C=C but a peak at 1415 $cm^{-1}$ may stand for C=C-H (18).

A further proof for the presence of a carboxylic functional group was the formation of the methyl ester of $X_2$. This was checked by thin layer chromatography (table 11) and gas-liquid chromatography (Fig.14). The peaks registered by the gas-liquid chromatograms supported the idea that $X_2$ is a $C_{16}$ compound.

Absorption in the ultra-violet region of the spectrum (Fig.12) confirmed that $X_2$, which registered a first band at 200-230 m$\mu$ and a diffused second band at 270-280 m$\mu$, has a ketonic functional group. Carboxylic

acids usually absorb in the ultra-violet region at one band namely 207-210 m μ; but such a wavelength cannot be properly detected under the given experimental conditions.

The formation of a 2,4-dinitrophenyl hydrazine derivative of $X_2$ gave more support for the presence of a ketonic group. This was proved by thin layer chromatography (table 11) and ultra-violet absorption spectrum (Fig.15). The absorption spectrum of $X_2$ phenyl hydrazone is typical of azo dyes (17).

The n.m.r. spectrum of $X_2$ (Fig.16) showed a triplet centered at $\tau$=4.67 (1H) attributed to vinyl protons, a doublet centered at $\tau$=7.68 (1H) assigned to a methylenic proton $\alpha$ to a carbonyl function, a large singlet at $\tau$=8.73 (28H) characteristic of aliphatic methylenic protons, and another singlet at $\tau$=9.10 (3H) assigned to one methyl group.

Although chemical tests for unsaturation namely the use of $Br_2$ water or $KMnO_4$ solution were negative thus indicating a saturated compound, yet such procedures would not reduce a C=C if it is a part of a vinyl group. However, the elementary chemical analysis and n,m.r.spectrum suggest the presence of at least 30-32 hydrogens.If the C=C is to be regarded as a part of $X_2$ and not a contaminant, the number of hydrogens can be regarded with a margin of ±2 hydrogens.A possible structure of $X_2$ would therefore be:

$$CH_3-(CH_2)_n-\overset{H}{C}=\overset{H}{C}-\overset{O}{C}-(CH_2)_{11-n}-\overset{O}{C}-OH$$

The empirical formula of such a compound is $C_{16}H_{28}O_3$ and its molecular weight is 268 which conforms within ±2 of the value of 270 obtained for molecular weight measurement.

A second possibility would be a saturated hydrocarbon with an empirical formula of $C_{16}H_{30}O_3$ and a molecular weight of 270. It may be pointed out here that while conducting studies on long-chain fatty acid oxidation in mammalian tissues, Jones et.al. (15) isolated 3-ketohexadecanoic acid $C_{17}H_{32}O_3$ ; 3-hydroxyhexadecanoic acid $C_{16}H_{32}O_3$ ; and 2-hexadecenoic acid $C_{16}H_{30}O_3$ .

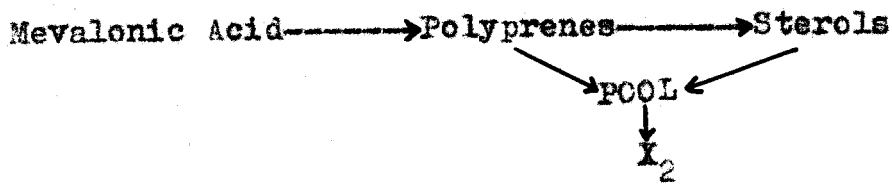
Some lipid-soluble substances with acidic properties, namely the prostaglandins, were isolated (19) from the homogenates of guinea pig lungs. A $C_{20}H_{34}O_5$ ,called $PGE_1$ , was identified as a carboxylic acid containing 2 hydroxyl groups, one keto group, one trans double bond, and a cyclopentanone ring. Arachidonic acid is usually converted to some prostaglandins (20) and these compounds are widely distributed in animal tissues.

Our findings that whatever stimulates the steroids formation stimulates the formation of $X_2$-like compounds, and whatever inhibits the steroids formation inhibits the formation of $X_2$ among other compounds, led us to speculate on how would mevalonic acid yield compounds such as $X_2$ . That is , what is the nature of the mechanism which involves the formation of such compounds derived from mevalonic acid and might function in the catabolic disposal of squalene precursors?

The pathway from mevalonic acid is far from being clear as to how we get $X_2$ formation. This is still opened to speculations and requires more detailed experiments. An impor-

tant point to be noticed here is the low radioactivity of $X_2$. If $X_2$ was a direct product of mevalonic acid or the polyprenes, its radioactivity would be expected to be higher; more sites of unsaturation are expected ; and more branching in its structure should occur. Hence, either a chain of reactions must be taking place whereby dilution occurs ;or, this radioactivity is a contamination by residual radioactive mevalonic acid.

Considering that $X_2$ is derived from mevalonic acid a possible pathway would be:




1. $X_2$ is derived from the polyprenes indirectly through a metabolic pool which might involve $CO_2$, acetyl CoA,etc. This pool is:
   a. Either a very large one thus accounting for the dilution of radioactivity in $X_2$, or
   b. It has a very fast rate of turn-over thus explaining the dilution of radioactivity in $X_2$.
2. $X_2$ might be derived from the sterols through several steps of cleavage.

## SUMMARY

A study was designed to isolate, purify and partially identify certain metabolites of mevalonic acid from rat liver. The pattern of these intermediates on column chromatography was examined, and the effects of time, pH and concentration on the metabolism of mevalonate were investigated. The effects of ascorbic acid, glutathione, cysteine, anaerobiosis and some inhibitors were studied as well.

Metabolites of mevalonate formed by minced rat liver were extracted, fractionated and purified by chromatography. $X_2$, one of the isolated metabolites, was purified and identified as a keto acid with an empirical formula of $C_{16}H_{28}O_3$. Evidence for the presence of a carbonyl group, carboxylic group and a possible site of unsaturation was given.

Preliminary studies indicate that mevalonic acid is undergoing conversion to one or more unknown compounds by a pathway wherein a redox system is operating.

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